MOLECULAR CHARACTERISATION OF THE PRP8 PROTEIN OF SACCHAROMYCES CEREVISIAE AND IDENTIFICATION OF AN ANALOGUE IN HELA CELLS

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ABSTRACT

In many respects the process of nuclear pre-mRNA splicing is similar in cells of the yeast, Saccharomyces cerevisiae, and of higher eukaryotes. The availability of yeast mutants which are deficient for this reaction provides the means to characterise components of the splicing machinery. The PRP8 gene has previously been cloned, by complementation of thegrowth defect of a strain carrying the prp8-1 temperature sensitive mutation, and has been shown to encode a 280kD protein which is required for pre-mRNA splicing. In this thesis, I describe the further analysis of this protein:

(i) Two gene fusions were constructed to allow synthesis of β-galactosidase fusion proteins containing regions of the PRP8 protein, not represented in previous constructs, which were used as antigens to raise antisera in rabbits. Both these antisera detected the 280kD protein in yeast cell extracts by immunoblotting, but only one was able to immunoprecipitate PRP8.

(ii) These, and existing, antisera were used to analyse the interactions of the PRP8 protein in yeast splicing extract. Using this immunological approach, it was demonstrated that the full-length PRP8 protein is stably associated with the spliceosomal U5 snRNA. Following incubation of the splicing extract in the presence of ATP, PRP8 becomes additionallly associated with U4 and U6 snRNAs, which normally exist together in a single snRNP particle. These data suggest that PRP8 is a component of the U5 snRNP particle and that this particle associates with the U4/U6 snRNP particle in an ATP-dependent manner.

(iii) Attempts were made to reconstitute the U5 snRNP particle in yeastextracts using radiolabelled U5 snRNA, transcribed in vitro from a synthetic gene. However, binding of snRNP-specific proteins could not be demonstrated, presumably due to competition by non-sequence specific RNA-binding proteins. A fusion protein containing part of the PRP8 protein was shown to bind RNA in a non-sequence specific manner by blot assay.

(iv) A 220kD HeLa cell protein was detected by antisera to three different fusion proteins and by affinity purified antibodies specific to the PRP8 portions of these

fusions. This species is present in purified human U5 snRNP fraction, is precipitable by anti-Sm antibodies and, as assayed on blots, binds RNA with no apparent sequence specificity. This provides suggestive evidence that this protein is a true homologue of the yeast PRP8 protein. In addition, preliminary evidence indicates the existence of a large cross-reacting protein in Drosophila melanogaster cells.

CONTENTS

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ABBREVIATIONS

A	:	Ampere
ATP	:	adenosine 5'-triphosphate
bp	:	base pair
BSA	:	bovine serum albumin
cDNA	:	complementary DNA
Ci	:	Curie ($2.2x10^{12}$ dpm)
CTP	:	cytosine 5'-triphosphate
D	:	Dalton
dATP	:	2'-deoxyadenosine 5'-triphosphate
dCTP	:	2'-deoxycytosine 5'-triphosphate
dGTP	:	2'-deoxyguanosine 5'-triphosphate
dTTP	:	2'-deoxythymidine 5'-triphosphate
ddATP	:	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	:	2',3'-dideoxycytosine 5'-triphosphate
ddGTP	:	2',3'-dideoxyguanosine 5'-triphosphate
ddTTP	:	2',3'-dideoxythymidine 5'-triphosphate
d/ddH_2O	:	distilled/double distilled water
DMSO	:	dimethyl sulphoxide
DNA	:	deoxyribonucleic acid
DNase	:	deoxyribonuclease
dpm	:	disintegrations per minute
DTT	:	dithiothreitol
EDTA	:	ethylene-diamine-tetracetic acid
g	:	acceleration due to gravity ($9.81 m.s^{-2}$)
g	:	gram
GTP	:	guanosine 5'-triphosphate
HEPES	:	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
hnRNP	:	heterogeneous nuclear RNP
IAA	:	indole acrylic acid
IgG	:	immunoglobulin G
IPTG	:	isopropyl β-D-thiogalactoside

k	:	kilo-(i.e. $x10^3$)
l	:	litre
m	:	milli-(i.e. $x10^{-}3$)
m	:	metre
M	:	moles/litre
m.o.i.	:	multiplicity of infection
MOPS	:	4-morpholine-propane-sulphonic acid
mRNA	:	messenger RNA
m^3G	:	2,2,7-trimethyl guanosine
M.Wt.	:	molecular weight
n	:	nano- (i.e. $x10^{-9}$)
NP40	:	Nonidet P-40
nt	:	nucleotide
$OD_{.650nm}$	:	optical density with respect to light of wavelength 650nm
PEG	:	polyethylene glycol
PMSF	:	phenyl-methyl-sulphonyl fluoride
psi	:	pounds per square inch
Pu	:	purine nucleotide
Py	:	pyrimidine nucleotide
RNA	:	ribonucleic acid
RNase	:	ribonuclease
RNasin	:	ribonuclease inhibitor
RNP	:	ribonucleoprotein
rpm	:	revolutions per minute
rRNA	:	ribosomal RNA
SDS	:	sodium dodecyl sulphate
SDS-PAGE	:	SDS-polyacrylamide gel electrophoresis
snRNA	:	small nuclear RNA
snRNP	:	small nuclear RNP
TCA	:	trichloroacetic acid
TEMED	:	N,N,N',N'-tetramethyl-ethylenediamine
Tris	:	2-amino-2-hydroxymethyl-propane-1,3-diol
tRNA	:	transfer RNA

Tween 20	:	polyoxyethylene(20)-sorbiton-monolaurate
UTP	:	uridine 5'-triphosphate
UV	:	ultra-violet light
V	:	Volt
v/v	:	volume per unit volume
W	:	Watt
w/v	:	weight per unit volume
μ	:	micro- (i.e. $x10^{-6}$)

Single Letter Amino Acid Code

A : alanine	M : methionine
C : cysteine	N : asparagine
D : aspartate	P : proline
E : glutamate	Q : glutamine
F : phenylalanine	R : arginine
G : glycine	S : serine
H : histidine	T : threonine
I : isoleucine	V : valine
K : lysine	W : tryptophan
L : leucine	Y : tyrosine

CHAPTER 1

INTRODUCTION

1.1 RNA SPLICING

Data suggesting that the coding regions of genes are not necessarily continuous, but can be interrupted by stretches of non-coding sequence, led to the proposal, in early 1977, that such sequences are removed from the transcribed RNA (reviewed in Witkowski, 1988). Since then, such intervening sequences, or introns, have been identified in eukaryotic genes encoding tRNA, rRNA, mRNA and snRNA, in archaebacterial tRNA genes and in eubacterial phage genes encoding mRNA. The non-intronic regions of the transcript are called exons and the cleavage and ligation reaction by which introns are removed is termed RNA splicing.

Introns, and the reactions by which they are excised, have been divided into four classes:

(i) those found in tRNA genes (Abelson, 1979),

(ii) the so-called Group I introns first described in the Tetrahymena rRNA, but also many fungal mitochondrial introns, chloroplast introns and bacteriophage T4 introns (Cech and Bass, 1986),

(iii) the Group II introns of fungal mitochondrial and chloroplast genes, with conserved sequences and proposed secondary structures distinct from those of Group I introns (Cech and Bass, 1986),

(iv) those found in nuclear protein-encoding genes (Green, 1986; Padgett et al., 1986; Sharp, 1987a).

This thesis is concerned with the machinery for the removal of introns from nuclear pre-mRNA and, accordingly, the introductory chapter will primarily constitute

an overview of the present state of understanding of this process. Its relationship to the other classes of splicing will be discussed in Section 1.10.

1.2 INTRONS IN NUCLEAR PROTEIN-ENCODING GENES

In higher eukaryotes, most nuclear protein-encoding genes are interrupted by at least one intron and, indeed, can contain as many as 50 (Wozney et al., 1981) and these can range in size from 31bp (Ghosh et al., 1978) up to approximately 60kb (Garber et al., 1983; Scott et al., 1983). In Saccharomyces cerevisiae, on the other hand, very few genes contain introns and only the MATa1 gene (Miller, 1984) has more than one. All those except the second intron of the MATa1 gene are located close to the 5' terminus of the pre-mRNA. Fink (1987) has suggested a mechanism whereby the yeast genome has lost its introns due to homologous recombination between chromosomal genes and cDNAs reverse transcribed from processed mRNAs, the mechanics of which would explain the retention of introns near the transcriptional start sites. The largest intron identified in yeast is 513bp (Leer et al., 1984).

Sequence comparisons of mammalian introns revealed homologies at the 5' and 3' splice sites (Breathnach and Chambon, 1981; Mount, 1982) and the consensus sequences for these elements are shown in Figure 1.1A. Natural (Weiringa et al., 1984) or synthetic (Rautmann et al., 1984) splice site consensus sequences were sufficient to direct accurate excision of unrelated viral or bacterial sequence placed between them.

In the known yeast introns, the splice site sequences are almost invariant (Guthrie et al., 1986: Figure 1.1A), but, unlike mammalian introns, deletional analysis revealed a requirement for an element in the 3' portion of the intron (Langford and Gallwitz, 1983; Pikielny et al., 1983). Sequence comparisons led to the suggestion that the absolutely conserved TACTAAC sequence located 20-60bp upstream of the 3' splice site was the essential element and this was confirmed by point mutational analyses (see Section 1.4.2).

1.3 MECHANISM OF THE SPLICING REACTION

While in vivo experiments allowed investigation of the importance of pre-mRNA sequence and structural elements for the splicing reaction, detailed analysis of the

FIGURE 1.1: INTRON STRUCTURE AND SPLICING MECHANISM

(A) Conserved sequence elements in pre-mRNA introns, where the 5' and 3' splice sites are indicated by vertical lines. Very highly conserved residues of metazoan introns are underlined, while the asterisk indicates the branch-point in yeast introns.

(B) Mechanism of nuclear pre-mRNA splicing in metazoa and S. cerevisiae.

A

METAZOAN:

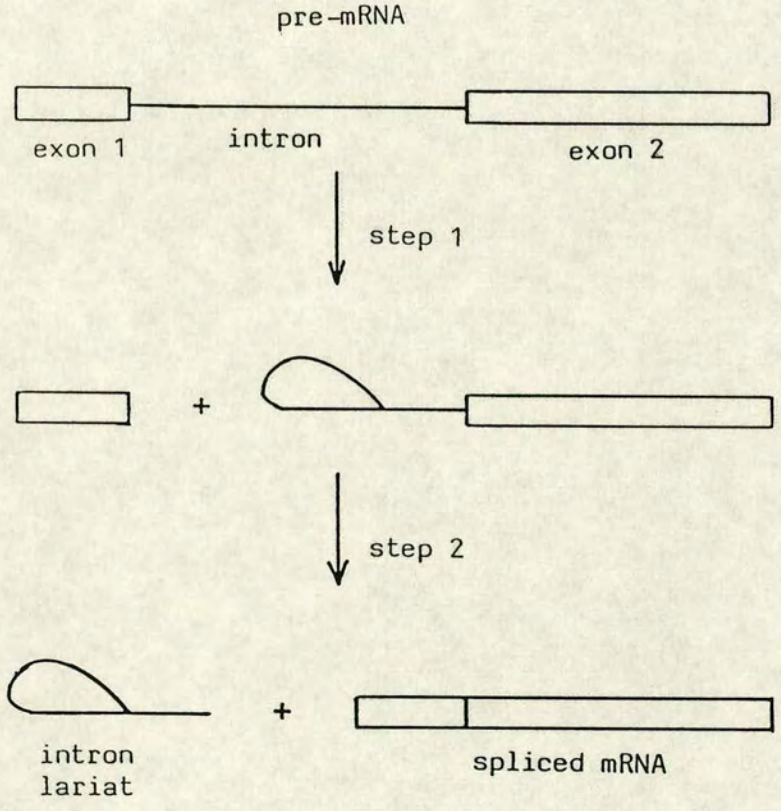
$\begin{matrix} C \\ A \end{matrix}$AG | GTAAGT----------(Py)$_n$NPyAG | G

S.CEREVISIAE:

| GTATGT----------TACTA$\overset{*}{A}$C--------AG |

←10-139bp→

B



mechanism and biochemistry of the reaction required the development of a cell-free in vitro system which would faithfully splice pre-mRNA. Early systems relied on coupled transcription and splicing and were therefore inefficient and allowed little scope for dissection (Weingartner and Keller, 1981; Kole and Weissmann, 1982; Padgett et al., 1983). The first uncoupled systems (Goldenberg and Raskas, 1981; Goldenberg and Hauser, 1983) employed, as substrate, poly(A)+ nuclear RNA and were therefore unsuitable for routine experiments. The development of systems which used HeLa cell nuclear extracts to splice in vitro synthesised pre-mRNA permitted the elucidation of the mechanism and biochemical requirements of the reaction (Hernandez and Keller, 1983; Hardy et al., 1984; Grabowski et al., 1984; Padgett et al., 1984; Krainer et al., 1984; Ruskin et al., 1984; Konarska et al., 1985a).

These studies resulted in the formulation of the two-step splicing pathway represented in Figure 1.1B. In each of the mammalian systems, a lag of approximately 30 minutes is observed before spliced mRNA can be detected, after which production of this species proceeds linearly. After the lag phase, the first detectable chemical change involves cleavage at the 5' splice site to generate the intermediates of the reaction, i.e. free exon 1 with a 3' hydroxyl group and intron-exon 2 in which the 5' end of the intron has formed a 2'-5' phosphodiester bond with an adenosine residue near the 3' end of the intron, a structure termed a "lariat". This constitutes "step 1" of the reaction.

The next stage of the reaction, "step 2", involves cleavage of the phosphodiester bond between the intron and exon 2 and ligation of the two exons to give the spliced mRNA product and the excised intron, still in lariat form, with a 3' hydroxyl group. Although ATP is strictly required for the in vitro splicing reaction, neither step 1 nor step 2 results in a net change in the number of phosphodiester bonds and so they could, theoretically, proceed without input of energy.

If the events observed in the in vitro splicing reaction reflect the in vivo process, then equivalent intermediate and product species should be detectable in mammalian cells. It was shown that polyadenylated hnRNA from HeLa cells contains branched molecules (Wallace and Edmonds, 1983), while analysis of β-globin transcripts from rabbit foetal liver cells (Zeitlin and Efstradiatis, 1984) revealed a series of species, some of which correspond to the lariat forms observed in vitro for the same transcript (Ruskin et al., 1984).

Lariat structures were also identified as in vivo products of the pre-mRNA splicing reaction in Saccharomyces cerevisiae and the branch-point was mapped to the last adenosine residue of the TACTAAC sequence (Domdey et al., 1984; Rodriguez et al., 1984). Subsequent development of a yeast in vitro splicing system (Lin et al., 1985) revealed an ATP-dependent, two-step pathway analagous to that in mammalian splicing.

With the finding that in yeast the 2'-5' phosphodiester bond forms within a highly conserved sequence element, a conserved branch-point sequence was sought in metazoan introns. By searching for homology to the yeast TACTAAC element in introns from a wide range of higher eukaryotes, the consensus sequence PyTPuAPy was formulated (Keller and Noon, 1984; 1985). Analysis of lariat-containing RNA species generated during in vitro splicing of a wide range of substrates revealed that branch formation generally occurs at an adenosine residue within a sequence conforming to this consensus (Reed and Maniatis, 1985).

1.4 ANALYSIS OF PRE-mRNA SEQUENCE AND STRUCTURE REQUIREMENTS

1.4.1 Mammalian Pre-mRNA

Naturally occurring mutations in the 5' splice site of the first intron of the human β-globin gene result in activation of cryptic 5' splice sites both in vivo (Treisman et al., 1983) and in vitro (Krainer et al., 1984). Point mutational analysis of the β-globin 5' splice site revealed that, in general, deviation from the consensus sequence led to a decrease in the efficiency of utilisation of the site (Aebi et al., 1986; Eperon et al., 1986; Aebi et al., 1987). Several of the mutations permit step 1 of the splicing reaction to occur, while abolishing step 2 (Aebi et al., 1987).

Deletion of the 3' splice site results in a complete abolition of splicing, both in vivo and in vitro (Reed and Maniatis, 1985). There are conflicting reports on the requirement for the highly conserved AG dinucleotide for step 1 of the splicing reaction, indicating either that it is dispensible (Ruskin and Green, 1985a) or important (Aebi et al., 1986) for this step. The importance of the polypyrimidine tract is demonstrated by the finding that progressive reduction of the size of this element results in a reduction in splicing efficiency (Weiringa et al., 1984; van Santen and Spritz, 1985).

Mutation of this region leads to a complete block in step 1 of the reaction (Frendeway and Keller, 1985; Ruskin and Green, 1985a).

Deletion of the authentic branch-point region results in the utilisation of cryptic sites, with little apparent sequence requirement but, seemingly, with a strong distance constraint in that they all occur between 22 and 37 nucleotides upstream of the 3' splice site (Ruskin et al., 1985). The branch usually forms at an adenosine residue, but mutations at the branch nucleotide reveal that, although an adenosine residue is favoured, any nucleotide can act as the branch acceptor (Hornig et al., 1986). However, lariats branched at a uracil or guanosine residue do not undergo step 2 of the reaction (Hornig et al., 1986). Taken together with the results obtained with point mutations of the first nucleotide of the intron (see above) these results indicate a requirement for specific nucleotide residues on either side of the 2'-5' phosphodiester bond in order for step 2 to take place. Reed and Maniatis (1988) set up an in vivo cis-competition assay to determine the importance of the surrounding sequence in branch-point selection. By the removal of all possible cryptic sites, they were able to demonstrate that the conserved nucleotides around the branch-point do, indeed, play a role in its selection and utilisation.

Synthetic consensus splice sites are only utilised when inserted in close proximity to authentic splice sites, thus implying a role for the "context", be it primary or secondary structure, of the splice site in its selection (Nelson and Green, 1988). Similarly, deletion, substitution, or even subtle changes in adjacent exon sequences were found to alter the efficiency of selection of a splice site (Reed and Maniatis, 1986).

The minimum intron size requirement for splicing was shown, by deletional analysis, to be in the order of 80 nucleotides (Weiringa et al., 1983; van Santen and Spritz, 1985). A 5' exon of 20nt is sufficient to allow splicing (Parent et al., 1987), while a pre-mRNA lacking a 3' exon can undergo step 1 (Frendeway and Keller, 1985) and one with as little as 4nt can be spliced (Turnbull-Ross et al., 1988).

The cap structure, $m^7G(5')ppp(5')N$, which is added to pre-mRNA in vivo, is not essential for splicing of an exogenous substrate in a HeLa cell nuclear extract, but does improve the efficiency of the reaction (Krainer et al., 1984). Splicing in vitro does not require 3' cleavage or polyadenylation of the pre-mRNA (Krainer et al., 1984).

1.4.2 Yeast pre-mRNA

Unlike the case in mammalian pre-mRNA, deletion of the authentic 5' splice site of a yeast intron does not result in activation of cryptic sites (Gallwitz, 1982). Point mutation of either of the first two nucleotides generally permits step 1 to occur, but not step 2 (Newman et al., 1985; Fouser and Friesen, 1986; Vijayraghavan et al., 1986), while mutation of position 5 results in an aberrant 5' cleavage event, upstream of the authentic site (Parker and Guthrie, 1985; Fouser and Friesen, 1986; Vijayraghavan et al., 1986). The aberrant cleavage sites have no similarity to the authentic site and, therefore, it seems that the cleavage event is separate from the 5' splice site recognition event and that the invariant guanosine residue at position 5 is important for this.

Deletion of the branch-point sequence generally leads to a block in splicing (Langford and Gallwitz, 1983; Langford et al., 1984; Pikielny et al., 1983), although, in the case of the actin transcript, a cryptic branch-point sequence, UACUAAG, is utilised (Cellini et al., 1986a). Point mutations within the conserved branch-point sequence inhibit splicing to varying extents (Langford et al., 1984; Newman et al., 1985; Vijayraghavan et al., 1986; Fouser and Friesen, 1986; Jacquier et al., 1985; Jacquier and Rosbash, 1986). When the branch-point adenosine nucleotide is mutated, either splicing is completely abolished (Langford et al., 1984; Newman et al., 1985) or step 1 and a low level of step 2 can take place (Fouser and Friesen, 1986; Jacquier and Rosbash, 1986; Vijayraghavan et al., 1986). The conflicting nature of these data may reflect the different pre-mRNAs used or differences in the sensitivity of the detection methods. However, it is clear that the branch-point is fixed as the penultimate residue of the sequence element and that, when a branch is formed at a mutated residue in this position, step 2 is severely inhibited. As is the case in mammalian introns, therefore, the residues linked by the 2'-5' phosphodiester bond are important for the second, rather than the first, step of the splicing reaction.

When the 3' splice site AG dinucleotide is deleted, the first AG downstream of the branch-point is utilised as an alternative site (Langford and Gallwitz, 1983). A transcript lacking a downstream AG can still undergo step 1 of the reaction (Rymond and Rosbash, 1985), provided that it extends at least 29nt beyond the branch-point sequence (Rymond et al., 1987), while, for step 2, the AG dinucleotide is important (Vijayraghavan et al., 1986; Fouser and Friesen, 1987) and the distance between the

branch-point sequence and the 3' splice site must lie between 8 and 66nt (Fouser and Friesen, 1987; Cellini et al., 1986b).

A role for non-conserved sequences of certain yeast introns has been suggested by the finding that deletion of a region upstream of the branch-point sequence of the CYH2 intron leads to an inhibition of splicing (Newman, 1987). Further deletion of a region near the 5' end of the intron restores splicing, suggesting the presence of an inhibitory element in this region which must be complemented by the 3' element or deleted to allow efficient splicing. In the case of the rp51A transcript, deletion of a non-conserved region near the 5' splice site leads to a reduction in splicing efficiency (Pikielny and Rosbash, 1985).

Enlarging the actin intron leads to a reduction in the efficiency of its removal from pre-mRNA (Klinz and Gallwitz, 1985), while a reduction of the distance between the 5' splice site and the branch-point to less than 44nt abolishes splicing (Thompson-Jäger and Domdey, 1988). The shortest natural introns are those in the MATa1 transcript with 43nt (intron 1) and 42nt (intron 2) separating the 5' splice sites and the branch-points (Miller, 1984) and removal of 6nt from intron 1 completely abolishes splicing (Köhrer and Domdey, 1988).

The location of almost all yeast introns near the 5' end of the pre-mRNAs might suggest a preference for a short first exon and, indeed, it was shown that moving the intron further from the 5' end of the actin transcript resulted in a reduction in the efficiency of, but did not abolish, splicing (Klinz and Gallwitz, 1985). The shortest first exon which supports splicing is 12nt, although one consisting of only one nucleotide allows step 1 to take place (Duchene et al., 1988), while a downstream exon of 10nt is sufficient (Rymond and Rosbash, 1985).

Neither a cap structure nor a poly(A) tail is required for the efficient splicing of exogenous substrate in a yeast in vitro splicing system (Lin et al., 1985).

1.4.3 Comparison of Mammalian and Yeast Requirements

The major contrast between the recognition of yeast and mammalian introns appears to be in the relative importance of the branch-point and 3' splice site conserved sequences. In mammalian introns, the polypyrimidine tract is essential for step 1 of the reaction and the branch-point appears to be determined, at least in part, by its distance

upstream of the 3' splice site. However, the degree of match to the consensus branch-point does play a role in the efficiency of utilisation of a mammalian branch-point, so clearly this element must be directly recognised by a component of the splicing apparatus. In yeast introns, on the other hand, recognition of the branch-point UACUAAC sequence appears to constitute an early event and the position of the 3' splice site is determined as the first AG dinucleotide downstream of this element.

The strict requirement for the UACUAAC element for recognition of introns in yeast is reflected in the finding that mammalian introns, which do not carry this sequence, are not spliced in yeast cells (Beggs et al., 1980). Conversely, the yeast rp51A transcript is spliced in HeLa cell nuclear extract, but with utilisation of a different branch-point (Ruskin et al., 1986), illustrating the differing mechanisms of branch-point selection between these organisms.

Although cryptic 5' splice site activation is not observed in yeast, mutations at the first two nucleotides of the intron have similar effects in mammalian and yeast introns. The effect of mutation of position 5 of the yeast sequence, i.e. shifting the 5' cleavage event to a position with no match to the consensus, has not been seen with mammalian introns.

1.5 SMALL NUCLEAR RIBONUCLEOPROTEIN PARTICLES

1.5.1 Mammalian snRNPs

1.5.1.1 Introduction

Small nuclear ribonucleoprotein particles are present in the nuclei of all eukaryotic cells so far analysed and consist of a snRNA moiety complexed with a set of polypeptides (reviewed in Steitz, 1988). The major species, U1, U2, U4, U5 and U6, range in size from 106-189nt, are abundant (2×10^5 -1×10^6 copies/cell) and, with the exception of U6, possess a trimethylguanosine cap. The U4 and U6 species are found in a single particle, base-paired to one another (Hashimoto and Steitz, 1984; Bringmann et al., 1984). The analysis of the structure and function of these particles has been facilitated by the availability of certain antibodies against their components. Antibodies against trimethyl guanosine recognise the cap structure and so can be used to precipitate

all these species, including U6 via its interaction with U4 (Bringmann et al., 1983; Smith and Eliceiri, 1983). Sera from human patients with certain autoimmune diseases recognise all the major snRNPs (anti-Sm), U1 only (anti-(U1)-RNP), U2 only (anti-(U2)-RNP), or U1 and U2 (anti-(U1,U2)-RNP) through their protein components (reviewed in van Venrooij, 1987).

1.5.1.2 Elucidation of Role in Splicing

The first proposals that the U-snRNPs are involved in pre-mRNA splicing were based on the sequence complementarity between the highly conserved 5' terminus of the U1 snRNA and the 5' and 3' splice site consensus sequences and suggested that the U1 snRNP might act to align the two splice sites for the splicing reaction (Lerner et al., 1980; Rogers and Wall, 1980). Subsequently, evidence accumulated for the involvement of U1 snRNP in splicing and, particularly, in recognition of the 5' splice site. Splicing in isolated HeLa cell nuclei (Yang et al., 1981) and in HeLa whole cell extract (Padgett et al., 1983) was inhibited by both anti-Sm and anti-(U1)-RNP sera and inhibition of in vivo splicing by these same two types of antisera was demonstrated by microinjection into oocytes of Xenopus laevis (Bozzoni et al., 1984; Fradin et al., 1984).

Analysis of protected RNA fragments after immunoprecipitation with anti-(U1)-RNP antibodies and RNase T1 digestion showed that the U1 snRNP binds the 5' splice site of pre-mRNA and that the association depended upon the integrity of both the snRNA and the protein components of the snRNP (Mount et al., 1983). Selective removal of the 5' end of the U1 snRNA, by treatment with a complementary oligonucleotide and RNase H, abolished splicing in an in vitro system (Krämer et al., 1984; Krainer and Maniatis, 1985).

Direct genetic evidence for the recognition of the 5' splice site by base-pairing with the 5' end of U1 snRNA was provided by the ability of compensatory mutations in a transfected U1 gene to suppress mutations in a 5' splice site in vivo (Zhuang and Weiner, 1986). However, suppression of one mutant was much more efficient than of another, suggesting that the 5' splice site sequence may be recognised by an additional factor. This is supported by the finding that U1 snRNP binds to synthetic and cryptic splice sites which are not utilised (Chabot and Steitz, 1987a; Nelson and Green, 1988). Therefore, base-pairing of the U1 snRNA is necessary, but not sufficient, for 5' splice

site selection. Analysis of the 5' splice site sequences of several thousand introns from evolutionarily diverse organisms revealed that, although the geometry of the interaction between the absolutely conserved 5' end of U1 snRNA and the 5' splice site was variable, the 5' cleavage always occurred opposite the junction between residues 9 and 10 of the snRNA (Jacob and Gallinaro, 1989). This suggests that the 5' cleavage site is determined by the interaction with U1 snRNP, and is supported by mutational analyses of the 5' splice site which showed that the cleavage site could be predicted from the most favourable base-pairing interaction with U1 snRNA (Aebi et al., 1987; Weber and Aebi, 1988).

The involvement of the U2 snRNP was indicated by the inhibition of the in vitro splicing reaction which results from RNase H-directed cleavage of the snRNA component (Black et al., 1985; Krainer and Maniatis, 1985), while immunoprecipitation with anti-(U2)-RNP antibodies followed by RNase T1 digestion revealed an interaction of the U2 snRNP with the branch-point region of the pre-mRNA (Black et al., 1985). This interaction requires the presence of a functional 3' splice site downstream of the branch-point region (Chabot and Steitz, 1987b). RNase H-directed cleavage of U4 and U6 snRNAs also revealed a requirement for the U4/U6 snRNP in splicing, but the U5 snRNA remained refractory to specific degradation (Black and Steitz, 1986; Berget and Robberson, 1986).

In protection assays, such as those which determined the sites of U1 and U2 snRNP interaction with the pre-mRNA, it was found that the 3' splice site was protected by a factor which was precipitable with anti-Sm and anti-m^3G antibodies, but was resistant to digestion by micrococcal nuclease (Chabot et al., 1985). Of the abundant Sm-precipitable snRNPs, only that containing U5 snRNA shows such resistance to micrococcal nuclease, giving rise to the suggestion that the U5 snRNP recognises and binds the 3' splice site.

1.5.1.3 Mammalian snRNP Proteins

The relative abundance of the major snRNP particles in mammalian cells, and the availability snRNP-specific sera, has facilitated their purification and the determination of their protein composition (Hinterberger et al., 1983; Kinlaw et al., 1983; Bringmann and Lührmann, 1986; reviewed in Lührmann, 1988). Each of the

major snRNP particles contains 7 common, "core" proteins (B', B, D, D', E, F, and G), while the U1 snRNP has 3 unique proteins (U1-70K, A and C) and the U2 snRNP has 2 unique proteins (A' and B"). A proportion of purified U5 snRNPs contains, in addition to the core proteins, up to 7 unique species (Bach et al., 1989). Cross-reactivity of rabbit sera raised against purified proteins, and similarities in peptide patterns after proteolytic digestion, has revealed that B and B' are closely related and that A and B" share homologous regions (Reuter et al., 1987). It is through direct recognition of subsets of core and specific proteins that the various autoimmune sera are able to precipitate snRNP particles (van Venrooij, 1987).

The snRNA species U1, U2, U4 and U5 each carries the so-called "domain A" motif, consisting of the sequence $PuA(U)_nGPu$ ($n>3$) in a single-stranded region flanked by double-stranded stems (Branlant et al., 1982). Intensive nuclease digestion of snRNP particles results in core protection of this site by the D, E, F, and G proteins (Liautard et al., 1982) and, indeed, this motif alone is sufficient to direct unrelated RNAs to become Sm-precipitable (Mattaj, 1986). The core snRNP proteins associate to form a 6S complex in the absence of snRNA (Fisher et al., 1985), suggesting that this subcomplex then recognises and binds the snRNA by virtue of the "domain A" structure. Only the F core protein can be covalently cross-linked to snRNA by UV-irradiation, implying that this species may play a role in binding directly to the "domain A" region (Woppmann et al., 1988).

The cDNAs encoding several of the snRNP proteins have been isolated and the sequences determined: U1-70K (Theissen et al., 1986), A (Sillekens et al., 1987), C (Sillekens et al., 1988), A' (Sillekens et al., 1989), B" (Habets et al., 1987), D (Rokeach et al., 1988), E (Wieben et al., 1985). The U1-70K, A and B" proteins contain regions of similarity to an 80 amino acid consensus sequence postulated to play a role in RNA-binding (Dreyfuss et al., 1988), and, for U1-70K, this motif has been shown to be a component of the U1 snRNA-binding domain (Query et al., 1989).

Analysis of the functions of the snRNP proteins will be facilitated by the development of in vitro reconstitution systems for the snRNP particles, as has been achieved for U1 (Patton et al., 1987; Hamm et al., 1987). This approach has allowed an analysis of snRNA-protein interactions and given rise to models for the overall structural arrangement of the U1 snRNP particle (Patton and Pederson, 1988; Hamm et al., 1988). It may be possible to assemble snRNP particles which are lacking a

particular protein and determine the effect on splicing in an in vitro system, thus providing clues to the function of the omitted species. Recently, the B and B" proteins have been shown to possess an endonucleolytic activity which is inactive in native snRNP particles (Temsamani et al., 1989). Whether this is functionally important for splicing is not clear, as it may play a role in snRNA maturation or in snRNP turnover.

Two different studies have identified a protein, with an apparent molecular weight of 100kD (Tazi et al., 1986) or 70kD (Gerke and Steitz, 1986), which binds the 3' splice site region of pre-mRNA. The 70kD species probably represents a degradation product of the 100kD species, reflecting the different purification procedures. This intron-binding protein (IBP) co-purifies with the U5 snRNP, under certain conditions, and is precipitable with the monoclonal anti-Sm antibodies used by Gerke and Steitz (1986). The binding of this factor, which occurs at 0°C in the absence of ATP, may result in the 3' splice site protection seen under such conditions and if associated with the U5 snRNP, would explain the precipitability of the protected fragment with anti-Sm and anti-m^3G antibodies (Chabot et al., 1985).

1.5.2 Yeast snRNPs

1.5.2.1 Identification of Yeast U-snRNAs

Several dozen snRNAs were identified in Saccharomyces cerevisiae, equivalent to the metazoan U-class on the basis of 5' cap structure, metabolic stability, nuclear localisation and presence of modified bases (Wise et al., 1983; Riedel et al., 1986). At less than 200 copies/cell, they are, however, considerably less abundant than the mammalian species. Many of these are not essential for cell viability (e.g. Parker et al., 1988), but the homologues of each of the mammalian species involved in pre-mRNA splicing have been identified and shown to be encoded by single-copy, essential genes.

The yeast U2 is 1175nt in length and, over 100nt at its 5' end, is similar in its primary sequence and predicted secondary structure to metazoan U2 snRNA (Ares, 1986). The reason for the enormous size discrepancy in U2 between yeast and metazoan cells is unclear, since approximately 950nt from the middle of the yeast species can be deleted without affecting cell viability (Igel and Ares, 1988; Shuster and Guthrie, 1988). Direct recognition of the branch-point sequence by base-pairing to U2 snRNA

was indicated by the ability of compensatory mutations in the latter to suppress the effects of mutations in the former (Parker et al., 1987). This suppression is not complete, indicating additional requirements on the branch-point sequence than base-pairing to U2 snRNA. These results, however, provide compelling evidence that base-pairing between the U2 snRNA and the pre-mRNA also plays a role in mammalian branch-point recognition.

The yeast U5 is present in two forms, of 179nt and 214nt, which are transcribed from the same gene and differ only in the presence or absence of a 35nt 3' extension (Patterson and Guthrie, 1987). The predicted secondary structure for these species is similar to that for the metazoan U5 snRNA and a 9nt region, which occurs in a predicted single-stranded loop, is absolutely conserved. The yeast U4 and U6 snRNAs are, like their metazoan counterparts, found in the same snRNP particle, base-paired to one another (Siliciano et al.,1987a; Brow and Guthrie, 1988). The yeast U4 snRNA exhibits considerable identity with the mammalian species over its first 68nt and the U6 species are 60% identical over their entire lengths, while the predicted base-pairing interaction is similar in both cases.

The yeast U1 snRNA, at 568nt, is considerably larger than its mammalian counterpart, with which it shares a perfect sequence match over its first 10nt and a similar predicted secondary structure (Kretzner et al., 1987; Siliciano et al., 1987b). That this species recognises the 5' splice site by a base-pairing interaction has been demonstrated by the partial suppression of mutations in the consensus sequence by compensatory mutations in the 5' end of the yeast U1 snRNA (Seraphin et al., 1988; Siliciano and Guthrie, 1988). However, restoration of base-pairing does not prevent the aberrant 5' cleavage events obtained with pre-mRNAs carrying mutations at position 5 of the 5' splice site consensus, nor does it restore step 2 of the reaction in pre-mRNAs with mutations at position 1 of the intron. This agrees with the findings in the mammalian system (Zhuang and Weiner, 1986) that base-pairing to U1 snRNA is necessary, but not sufficient, to define the 5' splice site.

1.5.2.2 Yeast snRNP Proteins

The relatively low abundance of snRNP particles in Saccharomyces cerevisiae means that their purification in sufficient quantities for protein analysis is very difficult.

The yeast U1, U2, U4 and U5 snRNAs all carry the "domain A" protein-binding site, as found in mammalian species, and are packaged into Sm-precipitable complexes upon injection into Xenopus oocytes (Tollervey and Mattaj, 1987; Riedel et al., 1987). In addition, the yeast splicing snRNPs are directly precipitable with certain anti-Sm sera (Tollervey and Mattaj, 1987; Siliciano et al., 1987; M.Dalrymple, personal communication). Taken together, these results indicate that there must be some conservation of snRNP proteins between yeast and mammalian cells.

The PRP8 protein, identified as being a pre-mRNA splicing factor by the genetic approach discussed in Section 1.6.2, has been shown to be a yeast snRNP component (this thesis; Lossky et al., 1987). Another protein, PRP4, appears to be associated specifically with the U4/U6 snRNP (Peterson-Bjorn et al., 1989; Banroques and Abelson, 1989).

1.6 OTHER PROTEIN FACTORS

1.6.1 Mammalian Factors

1.6.1.1 hnRNP Proteins

Pre-mRNA does not occur as naked nucleic acid, but rather as heterogeneous nuclear ribonucleoprotein (hnRNP), complexed with specific proteins as a linear array of globular, 200A monoparticles (reviewed in Dreyfuss et al., 1988). The first indication that such proteins play a role in pre-mRNA splicing was the finding that the hnRNP C-proteins were required for the in vitro splicing reaction (Choi et al., 1986). The C-proteins have a strong sequence preference for binding poly(U) (Swanson and Dreyfuss, 1988a) and, along with the hnRNP proteins A1 and D, have been shown to associate specifically with the polypyrimidine tract and branch-point sequences of pre-mRNA (Swanson and Dreyfuss, 1988b). Mattaj (1989) has proposed several possible roles for the hnRNP proteins in pre-mRNA splicing; (i) they may bind the intron to keep it free of unfavourable secondary structure or non-specific proteins, (ii) they may facilitate or stabilise adjacent protein binding, or (iii) they may block transport to the cytoplasm until splicing is complete.

1.6.1.2 Factors Identified by Fractionation

Chromatographic fractionation of crude HeLa cell nuclear extract has resulted in the definition of a number of splicing factors, necessary for either step 1 or step 2 (Krainer and Maniatis, 1985; Krämer and Keller, 1985; Furneaux et al., 1985; Perkins et al., 1986; Krämer et al., 1987). The lack of success in purifying such factors probably reflects the presence of several other essential factors in the fractions containing them.

A factor, U2AF, which is required for the binding of U2 snRNP to the pre-mRNA, has been identified and partially characterised, although its activity has proved too labile for purification (Ruskin et al., 1988). This factor is not hnRNP C-protein (Section 1.6.1.1) nor is it the intron-binding protein described in Section 1.5.1.3 and, in fact, binds independently of it. It binds pre-mRNA, requiring the polypyrimidine tract and the AG dinucleotide of the 3' splice site for efficient interaction. In a different study, two protein factors were shown to be essential for U2 snRNP interaction with pre-mRNA (Krämer, 1988). The relationship of these to previously described factors has not been fully investigated and the author points out that they may represent U2AF and intron-binding protein.

The results showing that base-pairing with U1 snRNA was not sufficient for selection of a 5' splice site (see Section 1.5.1.2), suggested the involvement of other factors in this recognition step. The existence of a protein factor which enhanced the binding of the U1 snRNP to the 5' splice site was suggested by fractionation experiments (Mayeda et al., 1986). A very simple, centrifugal fractionation procedure has revealed the requirement of a soluble, protein factor for the correct recognition of the 5' splice site by the U1 snRNP (Zapp and Berget, 1989).

1.6.2 Yeast Factors

In Saccharomyces cerevisiae, it is possible to define splicing factors genetically, as well as biochemically. The isolation of conditional-lethal mutants which are specifically defective in pre-mRNA splicing allows characterisation of the defect and isolation of the wild-type gene.

Of 400 mutants isolated for a heat-sensitive defect in macromolecular synthesis (Hartwell, 1967), 23, falling into 9 complementation groups (prp2-prp10/11), were

subsequently shown to accumulate pre-mRNA at the non-permissive temperature (Rosbash et al., 1981). Extracts from strains carrying these mutations, except prp6 and prp9, exhibit a higher degree of heat-sensitivity for the in vitro splicing reaction than do extracts from wild-type strains (Lustig et al., 1986), suggesting that the products of the mutated genes play a direct role in the reaction.

Several of the wild-type PRP genes have been isolated, selected from yeast genomic libraries on the basis of their ability to suppress the growth defects conferred by the mutations (Lee et al., 1984; Last et al., 1984; Soltyk et al., 1984; Jackson et al., 1988). The raising of antibodies to proteins produced from the cloned genes in E.coli provides the means to identify the protein products of the PRP genes (Lee et al., 1986; Last and Woolford, 1986; Chang et al., 1988; Peterson-Bjorn et al., 1989; Jackson et al., 1988) and to begin analysis of their roles in the pre-mRNA splicing reaction. In this way, the PRP8 and PRP4 proteins have been identified as components of snRNP particles (see Section 1.5.2.2) and the PRP11 protein has been shown to be a component of the spliceosome, but not stably associated with a snRNP (Chang et al., 1988). The PRP proteins could be purified by fractionation of a yeast splicing extract or by immunoaffinity chromatography, using complementation of heat-inactivated splicing extracts as assays of activity to monitor purification.

With the failure to develop a successful method for direct selection, the ways to isolate new prp mutations are; (i) to screen large banks of heat-sensitive mutants for accumulation of pre-mRNA, (ii) to isolate suppressors of the existing prp mutations, a method which has been used in this laboratory to isolate cold-sensitive suppressors of prp2 and prp8, or (iii) to isolate suppressors of mutations in conserved sequences of pre-mRNA which abolish splicing. This third approach has resulted in the isolation of a mutation, prp16, which allows splicing of a pre-mRNA carrying a branch-point sequence mutation (Couto et al., 1987). This gene encodes a factor distinct from U2 snRNA, which is, presumably, required in addition to U2 snRNA for branch-point recognition.

By fractionation of a yeast splicing extract, a factor essential for step 2 of the splicing reaction was defined (Cheng and Abelson, 1986). Subsequently, complementation studies on a heat-inactivated extract from a prp2 strain permitted the definition of two further yeast splicing factors, one required for step 1 and one for step 2 (Lin et al., 1987).

In extracts in which U2 snRNP was inactivated by RNase H-directed cleavage of its snRNA component, a factor was defined which commits the pre-mRNA to the splicing reaction upon complementation of the U2 depletion (Legrain et al., 1988). This factor binds stably to the pre-mRNA, in the absence of ATP, and requires the presence of intact 5' splice site and branch-point sequences. It cannot be excluded that this factor is, in fact, the U1 snRNP particle.

1.6.3 Schizosaccharomyces pombe

The genetic approach to definition of splicing factors is also available in the fission yeast Schizosaccharomyces pombe. Pre-mRNA splicing in this organism appears, in some ways, to be more characteristic of that in higher eukaryotes than is the process in Saccharomyces cerevisiae. Introns are common, with some genes having four (Hindley and Phear, 1984), the U-snRNAs are similar in size to those of metazoa (Tollervey, 1987; Brennwald et al., 1988) and a mammalian intron can be spliced in S.pombe (Kaufer et al., 1985), although this is not the general case because other mammalian introns are not spliced (Potashkin and Frendeway, personal communication). Three heat-sensitive S.pombe pre-mRNA splicing mutants have been isolated (Potashkin et al., 1989), but a full analysis of the products of these genes must await the development of a reliable in vitro splicing system.

1.7 SPLICING COMPLEXES

1.7.1 Mammalian Splicing Complexes

1.7.1.1 Identification and Composition

The association of splicing factors with the pre-mRNA results in the formation of a large ribonucleoprotein complex, in which the splicing reaction takes place. This was first identified by centrifugation of in vitro splicing reactions in glycerol gradients, when it was shown that, during the lag phase, the pre-mRNA is first formed into a 35/40S complex which then diminishes as a 50/60S complex is formed, where step 1 occurs (Frendeway and Keller, 1985; Grabowski et al., 1985). These first reports

indicated that only formation of the larger complex required ATP, but subsequently it was shown that formation of the smaller was also ATP-dependent (Steitz et al., 1988). The larger complex, which has been termed the "spliceosome", can be purified and, upon addition of nuclear extract, its pre-mRNA undergoes steps 1 and 2 without a lag phase (Grabowski et al., 1985). As expected, the spliceosome contains snRNPs as evidenced by its precipitability with anti-Sm antibodies (Grabowski et al., 1985).

By non-denaturing gel electrophoresis, the formation of two ATP-dependent splicing complexes could be detected (Konarska and Sharp, 1986). On the basis of the kinetics of their appearance, and of snRNP composition (see below), the fast and slow migrating complexes correspond to the 35/40S and 50/60S complexes respectively, and I will refer to these as A and B after the nomenclature of Konarska and Sharp (1986). Using a different substrate, it was possible to resolve complex B into two separate complexes (Lamond et al., 1987).

Splicing complexes have also been purified by chromatographic means. In two independent studies, biotinylated pre-mRNA was used as splicing substrate and then purified, along with associated factors, on streptavidin columns (Grabowski and Sharp, 1986; Bindereif and Green, 1987). In addition, spliceosomes have been purified by gel filtration chromatography and visualised in the enriched fractions by electron microscopy (Reed et al., 1988).

By using these approaches, or combinations of them, it has been possible to determine the snRNA components of the splicing complexes. Several such studies failed to detect U1 snRNA in any splicing complexes (Konarska and Sharp, 1986; Grabowski and Sharp, 1986; Lamond et al., 1987) which was surprising, since its role in 5' splice site binding was established. Also, the ability of anti-(U1)-RNP antibodies to precipitate splicing intermediates indicated that at least one component of this snRNP was still present after step 1 had occurred (Grabowski et al., 1985; Bindereif and Green, 1986; Chabot and Steitz, 1987a; Reed et al., 1988). It was subsequently shown that the conditions used in the earlier studies, i.e. high salt concentration and presence of heparin to compete non-specific RNA-binding, selectively dissociate the U1 snRNP from the splicing complexes. Therefore, under milder conditions, U1 snRNP could be detected in complexes A and B on sucrose gradients (Bindereif and Green, 1987), in gels (Zillmann et al., 1988) and in spliceosome fractions from gel filtration chromatography (Reed et al., 1988).

The U2 snRNA is present in complexes A and B (Grabowski and Sharp, 1986; Bindereif and Green, 1987; Konarska and Sharp, 1986), while U4, U5 and U6 snRNAs are present in B, but not A (Grabowski et al., 1986; Bindereif and Green, 1987; Konarska and Sharp, 1987). These results suggest that U1 and U2 snRNPs are present in complex A and that, in the transition to complex B, U5 and U4/U6 snRNPs join the forming spliceosome.

When complex B can be resolved into two separate complexes, using β-globin substrate, it is found that the faster migrating of these contains pre-mRNA and U2, U4, U5 and U6 snRNAs, while the slower migrating contains splicing intermediates and lacks U4 snRNA (Lamond et al., 1988). This suggests that U4 snRNP leaves the spliceosome just prior to, or concomitant with, step 1 of the reaction. However, since U1 snRNA cannot be detected in complexes in this gel system, it is possible that, in the active complex, the U4 snRNP is present, but can be dissociated under the electrophoresis conditions. However, these results show, at the very least, that some conformational change takes place just prior to, or at the same time as, step 1.

1.7.1.2 Splicing Complex Formation

With respect to the pre-mRNA sequence requirements for spliceosome formation, it has been shown that the polypyrimidine tract of the 3' splice site is essential (Frendeway and Keller, 1985; Bindereif and Green, 1986), while the AG dinucleotide is not necessary for, but enhances the efficiency of, complex formation (Frendeway and Keller, 1985). Early studies indicated that complex A could form in the absence of a 5' splice site (Frendeway and Keller, 1985; Konarska and Sharp, 1986), but it was later shown that the complex formed on such a substrate can be resolved as migrating faster on a non-denaturing gel than authentic complex A and, therefore, presumably lacks factor(s) present in complex A (Zillmann et al., 1988). Analysis of complex formation on substrates with point mutations at both 5' and 3' splice sites reveals that recognition of both is important for complex A assembly (Lamond et al., 1987).

As discussed in Section 1.5.1.2, the U1 snRNP binds to the 5' splice site region of the pre-mRNA. This interaction can occur at 0°C in the absence of ATP and, under splicing conditions, U1 is the first snRNP to associate stably with the substrate

(Bindereif and Green, 1987). Oligonucleotide-directed RNase H inactivation of U2 or U4 snRNPs has no effect on U1 binding, but immunodepletion of U1 snRNP inhibits subsequent binding of the other spliceosome snRNPs, indicating a key role for U1 snRNP binding in early complex assembly (Zillmann et al., 1987). However, U1 snRNP still associates with pre-mRNA which lacks a 5' splice site (Zillmann et al., 1987), suggesting that the initial interaction is not mediated by base-pairing between the U1 snRNA and the 5' splice site, but perhaps by recognition of sequences or factors at the 3' end of the intron. This idea is supported by the finding that formation of the A-like complex on substrates lacking the 5' splice site still requires the presence of intact U1 snRNP (Zillmann et al., 1988).

As was described in Section 1.5.1.2, the U2 snRNP binds to the branch-point region of the pre-mRNA in an ATP-dependent manner, this being a necessary step in complex A formation. Complex A formation is blocked by the directed cleavage of the 5' end of the U2 snRNA, but not by cleavage of the region proposed to base-pair with the branch-point sequence (Zillmann et al., 1988). This indicates that the base-pairing interaction is not necessary for complex A assembly, so U2 snRNP must recognise pre-bound U1 snRNP and factors at the 3' splice site, i.e. U2AF (see Section 1.6.1.2), IBP (see Section 1.5.1.3), hnRNP C-protein (see Section 1.6.1.1). Recognition of the branch-point by base-pairing must play a role in subsequent steps, as evidenced by the demonstration that certain point mutations in this sequence allow splicing complex assembly, but block step 1 of the reaction (Reed and Maniatis, 1988).

To assemble complex B, the U4/U6 and U5 snRNPs must join the growing spliceosome. The ATP-dependent formation of a U4/U5/U6 snRNP complex has been detected (Konarska and Sharp, 1987; D. Black, personal communication) and this may represent an intermediate in spliceosome assembly.

When step 2 of the splicing reaction has occurred, the products remain in ribonucleoprotein complexes, as assayed by centrifugation in sucrose gradients (Bindereif and Green, 1986). The spliced mRNA is released from snRNP-containing complexes, while the excised intron lariat appears to remain complexed with U2, U5 and U6 snRNPs (Konarska and Sharp, 1987; Zillmann et al., 1988).

Many studies have focussed on the protection of the pre-mRNA from degradation by RNases to analyse interactions with splicing factors through the course of splicing complex assembly and the splicing reaction (Black et al., 1985; Ruskin and

Green, 1985b; Chabot et al., 1985; Bindereif and Green, 1986; Chabot and Steitz, 1987b; Zillmann et al., 1988). Some of the results have been discussed in Section 1.5.1.2, whereby the 5' splice site is protected by the U1 snRNP and the 3' splice site factor by another, possibly U5 snRNP-associated, factor, both interactions occurring at 0°C in the absence of ATP. Also, the branch-point region is protected by the U2 snRNP in an interaction which requires incubation in the presence of ATP, i.e. splicing complex formation.

In the transition from complex A to complex B, changes in the nature of these interactions are indicated by changes in the boundaries of the protected branch-point fragment (Chabot and Steitz, 1987b) and by an expansion of the protected fragment at the 5' splice site (Chabot and Steitz, 1987b; Bindereif and Green, 1987). Directed cleavage of U4 and U6 snRNAs has no effect on the pattern of fragment protection, so this approach gives little indication of the possible roles of these particular snRNPs in the spliceosome (Chabot and Steitz, 1987b). Following step 1 of the reaction, a protected fragment from exon 1 is detected, presumably reflecting the binding of a factor which holds this intermediate in the spliceosome in order for step 2 to take place (Chabot and Steitz, 1987b).

1.7.2 Yeast Splicing Complexes

1.7.2.1 Identification

As in the mammalian case, yeast splicing complexes were initially identified by velocity sedimentation through glycerol gradients as 40S complexes which require ATP for formation and contain splicing intermediates (Brody and Abelson, 1985). They were subsequently analysed by non-denaturing gel electrophoresis (Pikielny and Rosbash, 1986), whereby they could be resolved into three complexes, termed I, II and III, in order of increasing mobility (Pikielny et al., 1986). Complex III is the first to appear, then complex I and, finally, complex II, where the splicing intermediates are found, suggesting that they represent a pathway of spliceosome assembly. Splicing complexes have also been isolated by affinity chromatography, relying on interaction between a poly(A) tail on the substrate and immobilised oligo(dT) (Pikielny and Rosbash, 1986)

or on biotinylated substrate interacting with immobilised streptavidin (Kretzner et al., 1987; Ruby and Abelson, 1988).

Analysis of the snRNA contents of the complexes observed on gels revealed that complex III contains only U2, complex I contains U2, U4 and U5, while complex II contains U2 and U5 (Pikielny et al., 1986). As for the analysis of mammalian splicing complexes on non-denaturing gel systems, U1 was not detected, but it was shown to be a component of affinity purified spliceosomes (Kretzner et al., 1987; Ruby and Abelson, 1988). Yeast U6 snRNA had not been described when the analysis of the complexes was undertaken, but it, also, was shown to be a component of spliceosomes (Ruby and Abelson, 1988). Therefore, all the yeast counterparts of the mammalian spliceosomal U-snRNPs are also components of the spliceosome. Moreover, the yeast U4 snRNP, like its mammalian homologue, appears to dissociate from the spliceosome just prior to, or at the same time as, step 1 of the reaction.

1.7.2.2 Yeast Splicing Complex Formation

All the mutations of the 5' splice site and branch-point sequences which abolish step 1 of the splicing reaction (see Section 1.4.2), also block spliceosome formation, while the 3' splice site is totally dispensible, as it is for step 1. A study which analysed the effects of chemical modifications, introduced at random in the pre-mRNA, on splicing complex formation revealed that the mutations in the conserved sequence elements can have effects at several levels of spliceosome formation and, therefore, suggested that these elements must be recognised more than once in the assembly process (Rymond and Rosbash, 1988).

The yeast U1 snRNP, like its mammalian homologue, is the first to stably bind the pre-mRNA, for which an intact branch-point sequence, but no ATP, is required, and this association is necessary for the subsequent ATP-dependent binding of the U2 snRNP (Ruby and Abelson, 1988). However, some pre-mRNA mutations which allow residual binding of U1 abolish U2 binding, so the binding of U1 snRNP is not sufficient, in itself, to direct the binding of the other snRNPs (Ruby and Abelson, 1988). The U2 snRNP binds the branch-point by direct base-pairing with its snRNA component (Parker et al., 1987), but this base-pairing interaction is not necessary for early complex

formation (Rymond and Rosbash, 1988). For the transition of complex III to complex I, the U5 and U4/U6 snRNPs join the spliceosome.

The 5' splice site and branch-point regions were found to be protected from oligonucleotide-directed RNase H cleavage in splicing complexes (Rymond and Rosbash, 1986). Interestingly, the degree of protection of the 5' splice site increases during the complex III to complex I/II transition, mirroring the results obtained in mammalian RNase protection studies (see Section 1.7.1.2).

1.8 CONTROL OF GENE EXPRESSION AT THE LEVEL OF PRE-mRNA SPLICING

Alternative splicing of a pre-mRNA occurs when at least one pair of splice sites which are spliced together to form one mRNA are not spliced in formation of another, i.e. each may remain unspliced or be spliced to an alternative partner. Each alternative mRNA may encode a protein product, or may be inactive, due to the presence of an in-frame termination codon.

In some cases, utilisation of alternative promoters or 3' end-processing sites results in pre-mRNA structures which dictate the subsequent choice of alternative splice sites. Such examples are beyond the scope of this discussion. Alternative mRNAs can be generated from a single pre-mRNA in several ways (reviewed in Breitbart et al., 1987); (i) retention or excision of a single intron, (ii) splicing of alternative 5' splice sites to a single 3' splice site, or vice versa, (iii) the incorporation of one or other of a pair of mutually exclusive exons in the mRNA, or (iv) the retention or omission of a single exon.

An example of regulated alternative splicing to produce different isoforms of the protein product is provided by the troponin T gene, where each of four short exons can be included or omitted and two further exons are mutually exclusive, giving rise, potentially, to 64 protein isoforms, in a developmental stage and tissue-specific pattern (Breitbart et al., 1985). The rat α-tropomyosin gene carries three pairs of mutually exclusive introns which are alternatively spliced to give mRNAs encoding the smooth or striated muscle or nonmuscle isoforms of the protein (Wieczorek et al., 1988).

Examples where the retention of a particular intron results in failure to produce a functional mRNA include the inhibition of splicing of a pre-mRNA by the protein

product of the spliced mRNA, i.e. feedback autoregulation, which is observed in the expression of certain ribosomal protein genes (Amaldi et al., 1989; Dabeva et al., 1986) and the suppressor-of-white-apricot gene of Drosophila melanogaster (Zachar et al., 1987; Chou et al., 1987). Such on/off control of gene expression can be regulated in a tissue-specific manner, as evidenced by the Drosophila P-element whose transcript carries three introns, the last of which is only excised, to give functional transposase-encoding mRNA, in germ-line cells (Laski et al., 1986).

Perhaps the most striking example of regulated pre-mRNA splicing occurs in Drosophila sex determination, where a cascade of alternative splicing connects the initial signal, i.e. the ratio of X chromosomes to autosomes, to final sexual differentiation events (reviewed in Hodgkin, 1989). Two of the genes involved in this regulatory pathway, sxl and tra, only give rise to active protein products in females, while the dsx gene gives rise to different proteins in males and females, all as a result of alternative splicing. The sxl gene product acts to regulate splicing of its own pre-mRNA and to confer female-specific splicing upon the tra gene pre-mRNA. The tra gene product acts, in turn, to regulate alternative splicing of the dsx gene pre-mRNA to give rise to the female-specific protein. In males, alternative splicing means that there is no active sxl gene product and, hence, no active tra gene product. This results in splicing of the dsx pre-mRNA such that the male-specific protein is expressed.

While alternative splicing could occur by an essentially random selection of splice sites, giving rise to a fixed ratio of different mRNAs, tissue or developmental stage-specific regulation of splicing must be controlled by the action of trans-acting factors. Such factors could act to specifically degrade certain alternative forms of mRNA or to modulate the selection of splice sites, either by altering the affinity of splicing factors for the alternative sites or by altering the pre-mRNA secondary structure, and hence the availability of alternative splice sites. Evidence that trans factors operate to modulate splice site selection is provided by the finding that only differentiated muscle cells give correct alternative splicing of troponin T pre-mRNA (Breitbart and Nadal-Ginard, 1987).

Candidates for splicing factors which could regulate alternative splicing include snRNAs, since the expression of different subclasses of U1 and U4 has been shown to be subject to developmental stage and tissue-specific control (Forbes et al., 1984; Lund et al., 1985; Lund et al., 1987; Lund and Dahlberg, 1987; Korf et al., 1988)). A snRNP

protein, termed N, which is closely related to the B and B' core proteins, is expressed only in brain and, to a lesser extent, heart tissues (McAllister et al., 1988; 1989). SnRNP particles formed with alternative snRNA or protein components could have modified recognition properties which change their affinities for alternative splice sites.

Analysis of cis-acting elements which can control splice site selection has been performed in vivo (e.g. Noble et al., 1988; Kedes and Steitz, 1988; Eperon et al., 1988), but attempts to reproduce the patterns of in vivo splice site selection in vitro have been unsuccessful (Noble et al., 1986; Lowery and van Ness, 1988). Identification and analysis of trans-acting factors requires that faithful, alternative splicing be achieved in vitro.

1.9 TRANS SPLICING

Trans-splicing is the process by which exons on distinct RNA transcripts, i.e. two "half-substrates", can be spliced together to give mRNA. That a mechanism might exist for such a reaction was first suggested by the finding that all mRNAs in the unicellular parasite Trypanosoma brucei share 35nt in common at their 5' termini (van der Ploeg, 1986). The sequences encoding this "leader" occur in repetitive clusters and are transcribed to give 137nt RNAs with the leader sequence at their 5' ends, followed by a consensus 5' splice site, while the bodies of each mRNA carry 3' splice site consensus sequences near their 5' termini. Branched intermediates were detected, which indicated that trans-splicing does, indeed, take place, and that it occurs by a two-step pathway similar to that of cis-splicing (Murphy et al., 1986; Sutton and Boothroyd, 1986). This process is not limited to trypanosomes, as a trans-spliced leader is also present on as many as 10% of the mRNAs of Caenorhabditis elegans (Blumenthal and Thomas, 1988) and on the mRNAs of other nematodes (Bektesh et al., 1988).

Two mechanisms for trans-splicing have been proposed. In the first, the half-substrates associate with one another by base-pairing between complementary regions within the "intron" and are spliced as for the normal cis-reaction. In vitro studies showed that such a scheme was viable (Solnick, 1985: Konarska et al.,1985b), but no sequence complementarity exists between the leader RNA and the region upstream of the 3' splice site on the mRNA body in trypanosomes or nematodes. Low levels of trans-splicing were observed in vitro in the absence of base-pairing between the RNAs,

indicating that physical attachment of the two half-substrates was not a pre-requisite of the reaction (Konarska et al., 1985b).

This led to the proposal that splicing factors could recognise, and bind, the two half-substrates independently and then coalesce to form the spliceosome (Sharp, 1987b). Support for this is provided by the finding that the C.elegans leader RNA forms into a snRNP particle, i.e. it carries a "domain A" Sm-binding site, is Sm-precipitable and carries a m^3G cap structure (Thomas et al., 1988; van Doren and Hirsch, 1988; Bruzik et al., 1988). Thus, factors binding the 3' half-substrate may be recognised and bound by the leader snRNP, carrying the 5' exon with it, in the formation of the "trans-spliceosome". In this way, the leader snRNP may replace the U1 snRNP in such a reaction (Bruzik et al., 1988). Support for this notion is provided by the failure to detect an analogue of U1 snRNA in T.brucei, where all mRNAs are formed by trans-splicing (Mottram et al., 1989).

1.10 PRE-mRNA SPLICING AND EVOLUTION

A comparison of the mechanisms of splicing of introns of nuclear pre-mRNA and Group I and Group II introns reveals striking similarities (Cech and Bass, 1986). All occur by two-step reactions in which the first step involves nucleophilic attack of the 5' splice site phosphodiester bond, by a guanosine nucleotide for Group I splicing and by the 2' hydroxyl group of a residue near the 3' end of the intron for Group II and nuclear pre-mRNA splicing. The intermediates are, therefore, free exon 1 and intron-exon 2, in a linear form for Group I splicing and as a lariat for the other two types. The second step involves nucleophilic attack of the 3' splice site phosphodiester bond by the hydroxyl group on the 3' end of free exon 1 to give spliced RNA and excised intron. These mechanistic similarities strongly suggest that these three types of intron are related and, therefore, evolved from a common ancestor.

There are examples of Group I and Group II introns which can undergo "self-splicing" reactions in vitro, i.e. without any requirement for protein. Since all introns of each type carry conserved primary and secondary structure elements, it is assumed that all such splicing occurs by an RNA-catalysed reaction, the rate of which is enhanced in vivo by the binding of proteins. The related nuclear pre-mRNA reaction may also occur by a process of RNA catalysis, where the catalytic RNA activity is

provided by snRNAs in the spliceosome, rather than, as for Group I and Group II intron self-splicing, by the intron itself. It is not difficult to envisage the evolutionary progression from a situation where the catalytic configuration is conferred by the secondary structure of the intron in association with specific proteins to one where such structures are provided in trans, i.e. by snRNPs. This limits the structural constraints on the intron to short conserved sequences, a situation which would greatly facilitate "exon shuffling" (see below).

The possible origin of introns has given rise to considerable debate, resulting in the postulation of two conflicting theories. The first holds that genes originally evolved with discontinuous structures, i.e. introns were present, and that they have been lost from certain organisms, especially prokaryotes, as their genomes have become more streamlined for rapid DNA replication (Doolittle, 1978). The second theory suggests that introns have become added to genes after the divergence of eukaryotes and prokaryotes and, therefore, that prokaryotic genes resemble ancestral genes (Orgel and Crick, 1980).

The revelation that RNA molecules act to catalyse certain reactions (reviewed in Cech and Bass, 1986; Cech, 1987) has led to the proposal that, prior to the evolution of protein synthesis, RNA served as both functional and informational molecule, i.e. the so-called "RNA world" (Gilbert, 1986). This theory states that self-splicing introns were an important component of such a world, acting, by trans-splicing, to allow RNA recombination. The discovery that the excised intron of Tetrahymena thermophila rRNA has enzymatic activity as a poly(C) polymerase (Zaug and Cech, 1986) led to the postulation of a scheme for RNA-catalysed replication of RNA (Cech, 1986) which lends considerable weight to the RNA-world theory. This model for pre-biotic evolution holds that genes originally evolved with discontinuous structures and that introns were subsequently eliminated.

Evidence that introns pre-dated the divergence of prokaryotes and eukaryotes has been provided by the discovery of introns in prokaryotic genes (reviewed in Schmidt, 1985) and from studies of chloroplast genes (Shih et al., 1988), while the elimination of introns has been well-documented (e.g. Perler et al., 1980). However, it is clear that certain introns can spread by DNA recombination (reviewed in Lambowitz, 1989) suggesting that the pattern of introns which exists today is the result of conflicting processes of insertion and elimination.

Several direct selective pressures may have played roles in the retention of introns in eukaryotic genes. Firstly, as discussed in Section 1.9, gene expression can be controlled at the level of splicing. Secondly, other steps in mRNA biogenesis, e.g. nuclear-cytoplasmic transport, have co-evolved with splicing and may, in some instances, be dependent upon it. Thirdly, certain introns may be retained because they contain important functional elements at the DNA level, e.g. enhancers or promoters (e.g. Bornstein and McKay, 1988). However, although the expression of some genes requires the presence of an intron (e.g. Buchman and Berg, 1988), the expression of others does not (Treisman et al., 1981; Ng et al., 1985).

One further postulated selective pressure to retain introns is the scope for assortment of exons provided by non-homologous recombination within introns which could serve to generate diversity during evolution (Gilbert, 1978; 1985). Support for such an "exon-shuffling" evolutionary mechanism is provided by the discovery that many exons correspond to functional or structural protein domains or modules (Gilbert et al., 1986) and that exons encoding related domains can be found in genes encoding unrelated proteins (Sudhof et al., 1985).

1.11 THIS THESIS

The PRP8 gene was cloned in this laboratory and the generation of antisera to β-galactosidase-PRP8 fusion proteins allowed identification of the protein gene product (Jackson et al., 1987). In this thesis, I present the following:

(i) generation and characterisation of antisera to further β-galactosidase-PRP8 fusion proteins.

(ii) demonstration that the full-length PRP8 protein is a component of the yeast U5 snRNP particle and that a U4/U5/U6 snRNP complex forms in yeast splicing extracts in an ATP-dependent manner.

(iii) analysis of the molecular interactions of the PRP8 protein, demonstrating putative RNA-binding properties.

(iv) identification of a PRP8 analogue in HeLa cell extracts, which is associated with the human U5 snRNP and also possesses RNA-binding properties.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Suppliers of Laboratory Reagents

Restriction enzymes and other DNA modifying enzymes:
Amersham International, Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs and Pharmacia.

Deoxyribonucleotides and dideoxyribonucleotides:
Pharmacia.

Ribonucleotides:
Sigma and Pharmacia.

Acrylamide and NN'-methylene bisacrylamide:
BDH Chemicals, "Electran" grade.

Agarose, Ultra-Pure Agarose and Low Melting-Point Agarose:
SeaKem and BRL.

Media reagents:
Difco Labs.

Standard laboratory reagents (analytical grade, or better):
BDH, Fisons, Sigma, May and Baker, Koch-Light, Bio-Rad and Serva.

2.1.2 Growth Media

All quantities listed are for 1 litre, unless otherwise stated. Solutions are autoclaved (see Section 2.2.2) and stored at room temperature. Antibiotic and amino acid stock solutions were added to media immediately before use.

2.1.2.1 E. coli Media

Luria broth (LB):	10g Bacto-Tryptone (Difco), 5g yeast extract (Difco), 10g NaCl, adjusted to pH7.2 with NaOH.
Luria-agar (LB-agar):	LB plus 15g agar (Difco).
LB/ampicillin:	LB plus 0.1mg/ml ampicillin.
Spizizen minimal agar:	2g $(NH_4)SO_4$, 14g K_2HPO_4, 6g KH_2PO_4, 1g Na_3 citrate.$3H_20$, 0.2g $MgSO_4$, 2g glucose, 2mg vitamin B1, 15g agar (Difco).
M9+CA medium:	6g $Na_2HPO_4.H_2O$, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl, 5g casamino acids (Difco), 2g glucose, 10mg vitamin B1, 0.25g $MgSO_4.7H_2O$, 22mg $CaCl_2.7H_2O$.
M9+CA+W medium:	M9+CA medium plus 20mg tryptophan.
Ampicillin stock:	0.1g/ml; stored at -20°C.

2.1.2.2 Yeast Media

YPDA:	10g Bacto-Tryptone (Difco), 20g Bacto-Peptone (Difco), 20g glucose, 20mg adenine sulphate.
YPDA-agar:	YPDA plus 20g agar (Difco).
YPDAS:	YPDA plus 216g sorbitol.
YMM:	6.7g yeast nitrogen base without amino acids (Difco), 20g glucose (supplemented with amino acids to 20mg/l as required).
YMM-agar:	YMM plus 20g agar (Difco).
amino acid stocks:	4mg/ml in dH_2O; filter sterilised and stored at 4°C.

2.1.3 Bacterial Strains

All strains were derivatives of E. coli K-12.

TABLE 2.1 Bacterial Strains

Strain	Genotype	Reference
HB101	F⁻, hsdS20(r_B^-,m_B^-), recA13 ara-14, proA2, lacY1, galK rpsL20(Sm^r), xyl-5, mtl-1, supE44.	Boyer and Roulland-Dussoix (1969)
DH5	F⁻, recA1, endA1, gyrA96, thiI, hsdR17(r_K^-,m_K^+), supE44.	Hanahan (1985)
BMH71-18	△(lac, pro), thi, supE, F'($lacI^q$, lacZ △M15, pro^+)	Messing et al. (1977)
pop2136	endA, thi, hsdR1,λcI^{857}.	K. Stanley (pers. comm.)
NM522	hsd△5, △(lac, pro), F'($lacI^q$, lacZ△M15, pro^+).	-

2.1.4 Yeast Strains

TABLE 2.2 Yeast Strains

Strain	Genotype	Source
BJ2412	a/α, ura3, leu2, trp1, gal2, pep4-3, prb1-1122, prc1-407.	E. Jones (Pittsburgh)
SPJ8.31	a, prp8-1, ura3-52, leu2, his3.	S. Jackson (this laboratory)

2.1.5 Plasmid Vectors and Constructs

TABLE 2.3 Plasmid and Phagemid Vectors

Vector	Description	Source	Reference
pEX-1 pEX-2 pEX-3	E. coli plasmids; multiple cloning sites in 3' end of lacZ gene, amp^R. For synthesis heat-inducible β-gal fusion proteins in strain pop2136.	-	Stanley and Luzio (1984).
pUR278 pUR288 pUR289 pUR290 pUR291 pUR292	E. coli plasmids; multiple cloning sites in 3' end of lacZ gene, amp^R. For synthesis of IPTG-inducible β-gal fusion proteins in strain BMH71-18 or NM522.	D. Lane (ICRF, Clare Hall)	Ruther and Muller-Hill (1983).
pATH-1 pATH-2 pATH-3	E.coli plasmids; multiple cloning sites in 3' end of trpE gene, amp^R. for synthesis of IAA-inducible TrpE fusion proteins.	D.Finnegan (Edinburgh)	Dieckmann and Tzogaloff (1985)
pEMBL8(+) pEMBL9(+)	E. coli phagemids; multiple cloning sites in segment of lacZ gene coding for α-peptide of β-gal; amp^R. F1 bacteriophage origin, allows synthesis of single-stranded phagemid DNA. Propagated in strain NM522.	N. Murray (Edinburgh)	Dente et al. (1983)
YEp24	intermediate copy number yeast plasmid; URA3; 2μm replication origin. E. coli plasmid; amp^R.	D. Botstein (M.I.T.)	Botstein (1979)

TABLE 2.4 Plasmid Constructs

Plasmid	Description	Source	Reference
pSP64-HB△6	pSP64 with human β-globin gene downstream of SP6 promoter.	A.Krainer (Harvard)	Krainer et al. (1984)
pSPrp51ApA	pSP62 with yeast RP51A gene (with region encoding poly-A tail) downstream of SP6 promoter.	C.Pikielny (Brandeis)	Pikielny et al. (1986)
pY8000	YEp24 with 9.0kb insert containing entire PRP8 gene.	S.Jackson (this lab)	Jackson et al. (1988).
pY85888	YCp50 with 7.6kb insert containing all but last 3 codons of PRP8 gene.	"	"
pY8712	pSPT18 with 1.3kb HindIII-BamHI fragment from 5' end of PRP8 gene.	"	S.Jackson (PhD thesis).
pFP8.1	pUR290 with 1.3kb BglII fragment of PRP8 gene.	"	Jackson et al. (1988).
pFP8.2	pUR290 with 2.4kb BglII fragment of PRP8 gene.	"	"

2.1.6 Miscellaneous Materials

2.1.6.1 Antisera

Antisera against FP8.1 (rabbits 46 and 47) and FP8.2 (rabbits 42 and 43) were raised by S. Jackson in this laboratory (Jackson et al., 1988).

Antibodies (purified by ammonium sulphate precipitation) against the m^3G cap structure were a gift from R. Lührmann (Marburg).

Human Sm-sera were provided by I. Mattaj (Heidelberg; Sm- "Kung"), D. Black (Yale; Sm-JE) and M. Dalrymple (this lab; Sm #24).

2.1.6.2 DNA Oligonucleotides

Yeast U2: 5'-CTACACTTGATCTAAGCCAAAAGGC-3'
Yeast U4: 5'-TTTCAACCAGCAAA-3'
Yeast U6: 5'-TC(T/A)TCTCTGTATTG-3'
Human U4: 5'-GGAAAAGTTTTCAATTAG-3'
Human U5: 5'-GACTCAGAGTTGTTC-3'
Human U6: 5'-GGCTTCACGAATTTGCGT-3'

DNA oligonucleotides were custom synthesised by the OSWEL DNA Service (Department of Chemistry, Edinburgh) or by the ICRF (Clare Hall) oligonucleotide synthesis service, except the oligonucleotide complementary to yeast U6 snRNA which was a gift from D.Brow (UCSF) and that complementary to yeast U2 snRNA which was a gift from D.Field (Toronto).

2.1.6.3 Others

Purified yeast polyadenylate-binding protein was a gift of A.Sachs (Stanford Medical School).

HeLa cell nuclear extract was provided by P. Sharp (M.I.T.).

2.2 GENERAL METHODS

2.2.1 General Guidelines

Unless otherwise stated all small-scale procedures were performed in 1.5ml polypropylene microfuge tubes (handled only with gloved hands and sterilised by autoclaving at 120°C, 15psi for 15 minutes), while large-scale procedures were performed in 15ml or 30ml Corex tubes (sterilised by baking at 250°C for 16 hours) or 10ml or 50ml Falcon tubes (sterile when purchased). Liquids were dispensed using Gilson Pipetman P-20, P-200 and P-1000 automatic pipettors and tips (sterilised as for microfuge tubes) or glass pipettes (sterilised as for Corex tubes). All procedures were performed at room temperature unless otherwise stated.

Centrifugation of volumes less than 1.5ml was performed in an Eppendorf microfuge at 13,000rpm (13,800g), centrifugation of volumes up to 50ml was performed in an IEC Centra-4X bench-top centrifuge with swing-out rotor, a MSE Minor 'S' bench-top centrifuge with fixed-angle rotor or a Sorvall RC-5B centrifuge with HB-4 swing-out rotor or SS-34 fixed-angle rotor (all at speeds specified for particular methods), while larger volumes were centrifuged in a Sorvall RC-5B centrifuge with GS-A or GS-3 rotors (speeds again as specified for particular methods). A Beckman OTD55B ultracentrifuge with a Ti50 rotor was used for ultracentrifugation.

2.2.2 Preparation and Sterilisation of Solutions

All solutions were prepared using distilled or double-distilled water and (for stock solutions) were filtered through Whatman No.1 filter-paper. Most solutions were sterilised, either by autoclaving at 120°C, 15psi for 15 minutes or by filtration through Acrodisc filter-sterilisation units (0.45μm pore-size; Gelman Sciences). Solutions were stored at room temperature unless otherwise stated.

2.2.3 Deionisation of Solutions

Solutions were deionised where necessary (as stated in particular methods) by treatment with 0.1 volume of mixed-bed ion-exchange resin (20-50 mesh, Bio-Rad

Laboratories) for 30 minutes at room temperature with stirring. The resin was then removed by filtration through Whatman No.1 filter-paper.

2.2.4 Autoradiography

[^{32}P]-labelled nucleic acids, in gels or on filters, were detected by exposure of X-ray film (Du Pont Cronex or Amersham Hyperfilm-MP) in light-proof lead-shielded cassettes by one of the following methods, depending on the amount of radioactivity in the gel or on the filter: (in order of increasing sensitivity) (i) at room temperature; (ii) at -70°C with a calcium-tungstate intensifying screen; (iii) with pre-flashed film at -70°C with an intensifying screen (Laskey, 1984).

[^{35}S]-labelled nucleic acids, in a fixed and dried gel, were detected by exposure of X-ray film (Cronex) at room temperature, without an intensifying screen.

[^{35}S]-labelled proteins, in a dried gel impregnated with Amplify, were detected by exposure to pre-flashed X-ray film (Cronex or Hyperfilm) at -70°C, without an intensifying screen.

2.2.5 Dialysis Tubing

Dialysis tubing (Medicell International Ltd.) was prepared by boiling in a large volume of 2% (w/v) sodium bicarbonate, 1mM EDTA for 10 minutes, rinsing thoroughly in dH_2O and boiling for 10 minutes in dH_2O and was stored at 4°C in 50% (v/v) ethanol. Before use, the tubing (which was always handled with gloved hands) was washed, inside and out, with dH_2O.

2.2.6 Buffers

TE buffer:	10mM Tris-HCl (pH7.5), 1mM EDTA; autoclaved.
10x TBE buffer:	0.9M Tris-borate (pH8.3), 20mM EDTA; unsterilised.
10x TAE:	0.4M Tris-acetate (pH7.5), 20mM EDTA; unsterilised.

20x SSC: 3M NaCl, 0.3M Na_3citrate, adjusted to pH7.0 with NaOH: unsterilised.

10x PBS: 15mM KH_2PO_4, 80mM K_2HPO_4, 1.5M NaCl, pH7.2; autoclaved.

10x TBS: 0.5M Tris-HCl, 1.5M NaCl, pH7.5; autoclaved.

2.3 MICROBIOLOGICAL METHODS

All procedures were performed under conditions of "Good Microbiological Practice" as recommended by the U.K. Genetic Manipulation Advisory Group.

2.3.1 Growth and Storage of E. coli

E. coli cells were grown at 37°C, except for transformed cells of strain pop2136 which were grown at 30°C. Untransformed strains HB101, DH5 and pop2136 were maintained on LB-agar plates and strains NM522 and BMH71-18 on Spizizen minimal agar plates. Transformed strains were maintained on LB/ampicillin-agar plates to select for plasmid-conferred resistance. Liquid cultures in LB or LB/ampicillin medium were continuously shaken and growth monitored by determination of the $OD_{.650nm}$. Cells in colonies on the surface of inverted agar medium plates remained viable at 4°C for up to 1 month, while long-term storage was achieved by making a saturated liquid culture 15% (v/v) in glycerol and freezing at -70°C.

2.3.2 Growth and Storage of Yeast

Yeast cells were propagated as described by Sherman et al. (1983). Temperature-sensitive prp8-1 strains were grown at 23°C, while all other strains were grown at 30°C. Untransformed strains were maintained on YPDA-agar plates, transformed strains on YMM-agar supplemented with the appropriate amino acids to allow growth and to select for retention of the plasmid. Liquid cultures in YPDA or

supplemented YMM medium were continuously shaken and growth monitored by determination of the $OD_{.650nm}$. Cells in colonies on the surface of inverted agar medium plates remained viable for at least 1 month at 4°C, while long-term storage was achieved by making a saturated liquid culture 15% (v/v) in glycerol and freezing at -70°C.

2.4 NUCLEIC ACID METHODS

2.4.1 Storage of Nucleic Acids

Double-stranded plasmid DNA was stored at 4°C, while single-stranded template DNA and DNA oligonucleotides were stored at -20°C.

2.4.2 Quantitation of Nucleic Acids

The $OD_{.650nm}$ of the nucleic acid solution was determined in a Perkin-Elmer 320 spectrophotometer and its concentration calculated by assuming that an $OD_{.650nm}$ of 1.0 corresponds to 50μg/ml of double-stranded DNA and to 40μg/ml of single-stranded DNA or RNA. Alternatively, the concentration of a double-stranded DNA solution was estimated by comparing the intensity of fluorescence of the unknown DNA with that of standard DNA solutions in an agarose mini-gel (see Section 2.4.6).

2.4.3 Deproteinisation of Nucleic Acid Solutions

Aqueous nucleic acid solutions were deproteinised by extraction with phenol and chloroform. An equal volume of phenol/chloroform was added to the nucleic acid solution and the phases mixed by vortexing (or, for larger DNAs, by gentle shaking). The phases were separated by centrifugation in a microfuge (13,800g) or Sorvall SS-34 rotor at 10,000rpm (12,000g) for 2-5 minutes and the upper (aqueous) phase transferred to a fresh tube. Extraction with phenol/chloroform was repeated as necessary (until no material was observed at the phase interface following centrifugation) and the procedure was then repeated with chloroform to remove any residual phenol. The nucleic acid

was subsequently ethanol precipitated (see Section 2.4.4) to remove any residual chloroform.

Phenol was redistilled and stored under dH_2O at -20°C. For extractions of DNA solutions phenol was pre-equilibrated with 1M Tris-HCl (pH8.0) and stored under TE buffer at 4°C. For extraction of RNA solutions phenol equilibrated with dH_2O was used. Chloroform was mixed 24:1 (v/v) with isoamyl alcohol and stored at room temperature.

2.4.4 Ethanol Precipitation of Nucleic Acids

To precipitate nucleic acid from an aqueous solution, 0.1 volumes of 3M sodium acetate (pH5.2) or 0.025 volumes of 4M NaCl and 2.5 volumes of ethanol were added and the mixture incubated at 4°C for >10 minutes or at -20°C for >1 hour. The precipitated nucleic acid was recovered by centrifugation in a microfuge (13,800g) or Sorvall HB-4 rotor at 10,000rpm (16,000g) for >15 minutes at 4°C. The pellet was washed by resuspending it in 70% ethanol and recovering by centrifugation as above. Following the wash step as much of the supernate as possible was drained off and the pellet was dried under vacuum. The nucleic acid could then be re-dissolved in the desired volume of the appropriate buffer.

2.4.5 Restriction Endonuclease Digestion of DNA

DNA was incubated with a greater than 2-fold excess of restriction endonuclease in the buffer recommended by the enzyme's manufacturer with the volume of the enzyme stock solution added not exceeding 10% of the total volume. Incubations were continued for at least one hour at the temperature recommended by the manufacturer. Reactions were terminated by heat-inactivation of the enzyme at 65°C for 10 minutes and/or by phenol/chloroform extraction.

2.4.6 Agarose Gel Electrophoresis of DNA

Horizontal 0.7-1.5% agarose slab gels were prepared by heating the agarose in TAE buffer to dissolve it, allowing it to cool to approximately 50°C and adding ethidium

bromide to 0.5μg/ml before casting the gel. 20x20x0.7cm submerged gels were used for preparative and most analytical purposes while 9x13x0.4cm gels with filter paper wicks were used for some analytical purposes. Before loading, 0.2 volumes of 30% glycerol, 0.25% bromophenol blue was added to the sample and the gel was run in TAE buffer at 5-20 V/cm. DNA was visualised on a 254nm (short wavelength) UV transilluminator and, if required, photographed through a red filter using Ilford HP5 5x4 inch negative or Polaroid 667 positive film.

2.4.7 Recovery of DNA Fragments from Agarose Gels

2.4.7.1 Electroelution

The following method is based on one described in Maniatis et al., (1982). For preparative gels, Ultra-Pure Agarose was used in order to reduce impurities which, if carried over in the recovered DNA, might impair subsequent reactions, e.g. ligation. For the purpose of this method, ethidium bromide was added to the electrophoresis buffer at 0.5μg/ml. After electrophoresis, DNA bands were visualised under a long-wavelength UV lamp (300-360nm) in order to minimise damage to the DNA. When the required fragment was well enough separated from any other fragments, the current was switched off and the buffer removed until it was below the level of the gel surface. Directly in front of the band of interest, a trough was cut in the gel, 5mm wider than the band on either side. A piece of dialysis tubing was inserted and pushed underneath the gel such that it formed a floor to the trough and a wall at the side furthest from the DNA band. The trough was filled with buffer from the tank and the current switched on. Under the UV light it was possible to observe the band eluting out of the gel into the trough and, when this was complete, the current was reversed briefly to release the DNA from the dialysis tubing. The current was switched off and the buffer from the trough recovered. This buffer, containing the eluted DNA, was phenol/chloroform extracted, ethanol precipitated and the purified fragment redissolved in TE buffer.

2.4.7.2 Ground Glass Method

DNA fragments were recovered from excised agarose gel pieces using the "GENECLEAN" kit (BIO 101 Inc.) precisely as dictated in the manufacturer's instructions.

2.4.8 Denaturing Acrylamide Gel Electrophoresis

Denaturing (sequencing-type) acrylamide gel electrophoresis of DNA and RNA was performed as described by Sanger and Coulson (1978). To give gels of the desired percentage polyacrylamide/7M urea in 1xTBE buffer, 9/10 volume stock solutions were prepared (e.g. 6.67% (w/v) acrylamide:bisacrylamide (20:1), 7.78M urea for a final gel of 6% polyacrylamide/7M urea), deionised, filtered and stored at 4°C. Gels were prepared by mixing 45ml of the stock solution with 5ml 10xTBE buffer, 0.4ml 10% (w/v) ammonium persulphate (freshly prepared) and 40μl of TEMED and pouring between two 42x23cm glass plates separated along their edges by 1x42cm strips of 0.4mm-thick modeller's Plastikard (Slater's Plastikard, Matlock, U.K.) and sealed with PVC tape. A Plastikard slot-forming comb (cut to give 1cm-wide wells) was inserted in the top of the gel and polymerisation was allowed to proceed for at least 1 hour. To run the gel, the PVC tape sealing the bottom edge of the plates was removed, the slot-forming comb withdrawn and the gel placed in the electrophoresis tank filled with 1xTBE buffer. After the gel was pre-electrophoresed for 30-60 minutes, the samples were denatured by heating to 90°C for 2 minutes in urea load buffer (10M urea, 1xTBE buffer, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol; the urea was deionised before the addition of the other components and the final mixture was filter sterilised and stored at -20°C) and loaded, using a drawn-out glass capillary. Electrophoresis was continued at 30-35W until the bromophenol blue and xylene cyanol marker dyes had migrated an appropriate distance.

2.4.9 Purification of DNA Oligonucleotides

DNA oligonucleotides were purified from denaturing polyacrylamide gels by electroelution. An aliquot of the crude oligonucleotide preparation containing 1.5-2.5

$OD._{260nm}$ units of DNA was dried down in a vacuum desiccator. The DNA was redissolved in 1μl of ddH_2O, 2μl of urea load buffer was added and the sample denatured and loaded onto a 1cm-wide well in a 0.8mm-thick 12% polyacrylamide/urea gel (see Section 2.4.8, the double thickness being achieved by the use of double side-spacers and comb). Electrophoresis was carried out at 30W until the bromophenol dye had run approx. 30cm, the gel plates were removed and the gel placed, on Saran Wrap, on a fluorescent thin-layer chromatography (t.l.c.) plate. Under a short-wavelength UV light, the DNA was visualised as dark shadows against the fluorescent background and the full-length species was excised using a scalpel. The DNA was recovered from the gel piece by elution in the "BioTrap" equipment (Schleicher & Schuell) in 1xTBE buffer at 200V for >1 hour. The sample was collected from the "trap", diluted to 1ml with dH_2O and desalted over a Sephadex G-25 column (NAP-10, Pharmacia). This involved pre-equilibrating the column with 2 bed volumes of 10mM potassium phosphate (pH7.5) followed by 2 bed volumes of dH_2O. The sample was loaded in a volume of 1ml, eluted in a volume of 1.5ml of dH_2O and dried down in the vacuum desiccator. The purified oligonucleotide was dissolved in 10μl of dH_2O and stored at -20°C.

2.4.10 Filling-in of Recessed 3'-termini of DNA

Recessed 3'-termini were filled using the DNA polymerising activity of the Klenow fragment of E. coli DNA polymerase I. The reaction was performed on the restriction fragment (up to 1μg of DNA) in 25μl of buffer as follows; 50mM Tris-HCl (pH7.5), 10mM $MgSO_4$, 0.1mM DTT, 50μg/ml BSA, 80μM of each of dATP, dCTP, dGTP and dTTP. 2 units of Klenow fragment were added before the reaction was incubated at 22°C for 15-30 minutes and terminated by heating to 70°C for 5 minutes to inactivate the enzyme.

2.4.11 Ligation of DNA

Vector and insert DNA fragments were mixed in the molar ratio 1:3 with the vector DNA at a concentration of approx. 10ug/ml for cohesive ends and in excess of 100μg/ml for blunt ends. This DNA mixture was ethanol precipitated and redissolved

in 10μl of ligation buffer (20mM Tris-HCl (pH7.6), 10mM $MgCl_2$, 10mM DTT and 0.6mM ATP with the addition of 15% PEG 6000 for blunt end ligations [Pheiffer and Zimmermann ,1983]). T4 DNA ligase (1-5 units) was added and the reaction incubated at 15°C overnight (for cohesive ends) or 22°C for 1 hour (blunt ends). A portion of the reaction mix was used directly for transformation of E. coli cells (see Section 2.4.12).

2.4.12 Transformation of E. coli Cells

2.4.12.1 Preparation of Competent Cells (except strain pop2136)

Transformation of E. coli strains, other than pop2136, was performed by a method based on that of Hanahan (1985). Cells were streaked onto a LB-agar plate and grown overnight at 37°C and a single colony was picked into 7ml of LB. This culture was grown with vigorous shaking at 37°C until it reached an $OD._{650nm}$ of approx. 0.3 and 5ml was then used to inoculate 100ml of LB and this second culture grown as above to an $OD._{650nm}$ of approx. 0.45 before being transferred to a pre-chilled 50ml Falcon tube and left on ice for 15 minutes. Cells were harvested by centrifugation in a IEC Minor 'S' bench-top centrifuge at 3,000rpm (1,000g) for 5 minutes at 4°C and washed once, by resuspension and centrifugation (as above), with TFBI (100mM RbCl, 50mM $MnCl_2$, 35mM sodium acetate, 10mM $CaCl_2$, 15% (v/v) glycerol (pH5.8); filter sterilised). The cells were collected by centrifugation, resuspended in 30ml of ice-cold TFBI, incubated on ice for 20 minutes, collected once more by centrifugation and resuspended in 4ml of TFBII (10mM MOPS (pH6.8, adjusted with KOH), 10mM RbCl, 75mM $CaCl_2$, 15% (v/v) glycerol; filter sterilised). After a further 20 minute incubation on ice the cells were competent for DNA uptake and were either used immediately or were snap-frozen in N_2(l) and stored for future use at -70°C.

2.4.12.2 Preparation of Competent Cells of Strain pop2136

Cultures were grown and harvested as for other strains (Section 2.4.12.1) and then resuspended in 30ml of ice-cold 100mM $CaCl_2$ and incubated for 1 hour on ice.

The cells were collected by centrifugation, resuspended in 1ml of ice-cold 100mM $CaCl_2$ and used immediately or stored for up to 24 hours on ice (but not frozen).

2.4.12.3 Transformation of Strains HB101, DH5, NM522 and BMH71-18

Aliquots of competent cells (0.2ml) prepared as above (Section 2.4.12.1) were mixed with 20-100ng of DNA, either ligation reaction mix (Section 2.4.11) or plasmid preparation (Section 2.4.14), and incubated on ice for 30 minutes. The mixture was transferred to a small glass test-tube and heated to 42°C in a water-bath with shaking for 2 minutes before being returned to ice for 1 minute. The mixture was diluted with 0.8ml of LB and incubated at 37°C on a rotating wheel for 1 hour. Aliquots of this transformed cell suspension were spread onto LB-agar/ampicillin plates and incubated overnight at 37°C. 10^4 to 10^7 transformants were obtained per 1μg of supercoiled pBR322 plasmid DNA used.

2.4.12.4 Transformation of Strain pop2136

DNA (20-100ng) was added to 0.1ml aliquots of competent cells prepared as above (Section 2.4.12.2) and incubated at 4°C for 10 minutes. The cells were heat-shocked at 34°C for 5 minutes before 0.9ml of LB was added. Incubation at 34°C was continued for 20 minutes before aliquots of the transformed cell suspension were streaked out on LB-agar/ampicillin plates and incubated overnight at 30°C. Up to 10^6 transformants were obtained per 1μg of supercoiled pBR322 plasmid DNA used.

2.4.13 Transformation of Yeast Cells

Yeast cells were transformed by the method of Ito et al. (1983). Cells were grown overnight in YPDA to stationary phase, at 23°C for temperature sensitive strains and at 30°C for others. 0.5ml of this culture was used to inoculate 50ml of YPDA and the culture grown at the appropriate temperature until the OD_{600nm} reached approx. 0.4 (about $8.5x10^6$ cells/ml). The cells were harvested by centrifugation in a 50ml Falcon tube in a Centra 4-X bench-top centrifuge at 3,000rpm (1,600g) for 5 minutes at room temperature. The cell pellet was resuspended in 5ml of TE buffer,

re-centrifuged as above, resuspended in a further 5ml of TE buffer, 0.208ml of 2.5M Li acetate was added to give a final concentration of 0.1M and the cells were incubated at the appropriate temperature (23°C or 30°C) for 1 hour with gentle shaking. DNA (1-10μg in 0.1ml) was added to 0.2ml aliquots of the cells and the incubation continued, without shaking, for 30 minutes before 0.7ml of 50% (w/v) PEG 4000 was added (to give a concentration of 35% (w/v)). The mixture was incubated for a further 1 hour at the same temperature as before and then heated to 42°C for 5 minutes. The cells were collected by centrifugation in a microfuge (13,800g) for 30 seconds at room temperature, resuspended in sterile H_2O, spun down again, resuspended in 0.1ml of sterile H_2O and streaked out on a YMM-agar plate supplemented to select transformants. The plates were incubated at 23°C or 30°C as appropriate and transformants were usually visible after 3 days.

2.4.14 Preparation of Plasmid DNA from E.coli Cells

2.4.14.1 Small-scale Rapid Alkaline Extraction of Plasmid DNA

This was performed by a method slightly modified from that of Birnboim and Doly (1980). Plasmid-carrying E.coli cells were grown overnight in 2ml of LB/ampicillin medium, 1.5ml of the culture was transferred to an Eppendorf tube and the cells spun down by centrifugation in a microfuge (13,800g) for 30 seconds at room temperature. The cell pellet was resuspended in 0.1ml of lysis solution (2mg/ml lysozyme, 10% (w/v) glucose, 25mM Tris-HCl (pH8), 10mM EDTA; freshly prepared) and incubated on ice for 30 minutes. The cells were then lysed by addition of 0.2ml of alkaline SDS solution (1% (w/v) SDS, 0.2M NaOH; freshly prepared) followed by incubation on ice for 5 minutes, then 0.15ml of 3M sodium acetate (pH4.8) was added and the incubation continued for a further 60 minutes on ice. Centrifugation in a microfuge (13,800g) for 5 minutes at room temperature was performed in order to spin out the cell debris and chromosomal DNA and the supernate was subsequently subjected to a phenol/chloroform extraction and ethanol precipitation. The DNA pellet was redissolved in 40μl of TE buffer containing 100μg/ml RNase A (1mg/ml stock solution prepared by boiling for 15 minutes to inactivate DNases and stored at -20°C) and incubated at 37°C for 30 minutes to digest RNA present in the preparation. This

procedure routinely yielded in excess of 1μg of plasmid DNA which was suitable for restriction analysis or cloning procedures.

2.4.14.2 Intermediate-Scale Alkaline Extraction of Plasmid DNA

This method constitutes a scaled-up version of that described for the small-scale preparation in Section 2.4.14.1. Cells were grown overnight as before but in a volume of 40ml and harvested by centrifugation in a Sorvall SS-34 rotor at 5,000rpm (3,000g) for 5 minutes. The procedure followed that described above except that the following volumes were used; 0.8ml lysis solution, 1.6ml alkaline SDS solution and 1.2ml sodium acetate (pH4.8). The cleared supernate (after centrifugation in a Sorvall SS-34 rotor at 12,000rpm [17,000g] for 5 minutes) was phenol/chloroform extracted, ethanol precipitated, the nucleic acid pellet redissolved in 0.4ml of TE buffer and 10μl of 1mg/ml RNase A added. After 30 minutes incubation at 37°C, 40μl of 3M sodium acetate (pH4.8) was added and the solution phenol/chloroform extracted and ethanol precipitated (centrifugation steps now being performed in a microfuge [13,800g]). The DNA pellet was finally dissolved in 0.1ml of TE buffer. This procedure yielded in excess of 20μg of plasmid DNA.

2.4.14.3 Large-scale CsCl Preparation of Plasmid DNA

This method was based upon that described by Guerry et al. (1973). Cells were grown overnight in LB/ampicillin medium and harvested in 200ml plastic bottles in a GS-A rotor at 5,000rpm (4,000g) for 5 minutes before being resuspended in 6ml of sucrose mix (25% (w/v) sucrose, 50mM Tris-HCl (pH8)) and transferred to 30ml polypropylene tubes. To this was added 1ml of lysozyme solution (10 mg/ml lysozyme in sucrose mix; freshly prepared) and the mixture was left at room temperature for 5 minutes before 1ml of 0.5M EDTA (pH8) was added. The tube was inverted several times, to allow mixing, then incubated at room temperature for a further 10 minutes. Cell lysis was achieved by the addition (by slow bubbling through the mix) of 10ml of ice-cold Triton mix (0.1% (v/v) Triton X-100, 50mM Tris-HCl (pH8), 62.5 mM EDTA (pH8)), standing on ice for 10 minutes and mixing gently. The lysate was cleared by centrifugation in a SS-34 rotor at 18,000rpm (39,000g) for 45 minutes at 4°C. Closed

circular plasmid DNA purification was achieved by equilibrium centrifugation in CsCl/ethidium bromide gradients as described by Maniatis et al. (1982). For each 1ml of cleared lysate, 0.95g of CsCl and 50μl of 10mg/ml ethidium bromide were added and the solution transferred to a Beckman 12ml polyallomer tube which was sealed and centrifuged in a Ti50 rotor at 37,000rpm (95,000g) for 48-60 hours at 18°C. The DNA was visualised using a long-wavelength UV lamp and the lower of the two bands (corresponding to closed circular plasmid DNA) was recovered by piercing the side of the tube with a 19-guage needle and syringe. Ethidium bromide was removed by repeated extractions with butan-1-ol (pre-equilibrated with CsCl-saturated TE buffer) and CsCl removed by dialysis against several changes of 2 litres of TE buffer over 16-20 hours at 4°C. The dialysate was subsequently phenol/chloroform extracted, ethanol precipitated and redissolved in 0.5-1.0ml of TE buffer. This procedure yielded anything from 0.1-1.0mg of plasmid DNA, depending on the particular plasmid and strain used.

2.4.15 Measurement of Radioactivity in Nucleic Acids

The incorporation of radiolabel into nucleic acids (see Sections 2.4.16 and 2.4.22) was determined by precipitation with trichloroacetic acid (TCA). A 1:10 dilution (in dH_2O) of the labelling reaction was made and 1μl of this was spotted onto a Whatman GF/C glass-fibre disc (2.1cm diameter), while a further 1μl was added to an Eppendorf tube containing 50μl of 0.5mg/ml E.coli tRNA. To this was added 13μl of 50% (w/v) TCA and the mixture was incubated on ice for 15 minutes before the precipitate was collected by filtering through a GF/C filter under a gentle vacuum. The filter was washed with 15ml of ice-cold 10% (w/v) TCA, followed by 15ml of ice-cold ethanol and then the radioactivity on each of the filters was determined by scintillation counting. The first filter measured the total radioactivity in the sample, while the second measured the radioactivity incorporated into nucleic acid, so comparison of the two values gave the percentage efficiency of the labelling reaction. Oligonucleotides less than 20 nucleotides in length are not quantitatively precipitated by this procedure.

2.4.16 Labelling of DNA Probes by Random-Priming

DNA isolated in a number of ways can be labelled for use as probes by the method of Feinberg and Vogelstein (1983). The method applies to the labelling of entire plasmid sequences (provided they were first linearised with a restriction endonuclease), purified DNA fragments, or, indeed, to DNA fragments separated from a mixture of fragments in a low melting-point (LMP) agarose gel (without the need for purification).

2.4.16.1 Purification of DNA Fragments from LMP Agarose Gels

A slab gel was prepared as described in Section 2.4.6, but using LMP agarose (BRL), and the DNA was electrophoresed with the running voltage not exceeding 7.5V/cm (to avoid smearing). The DNA was visualised on a UV transilluminator and the band containing the required fragment was excised. The gel piece was weighed, H_2O added (3ml per gram of gel) and the tube placed in a boiling water bath for 7 minutes to dissolve the agarose and denature the DNA. This solution can either be used immediately for the labelling reaction or stored at -20°C.

2.4.16.2 Labelling Reaction

Prior to commencing the labelling reaction the DNA must be completely denatured. This was achieved for DNA in LMP agarose by boiling as described above (but, if it had subsequently been stored, it was boiled for a further 3 minutes following thawing) while purified DNA fragment or linearised plasmid was boiled for 5 minutes. Reagents were added in the following order: dH_2O (to total volume of 50μl), 10μl oligo-labelling buffer (for preparation see below), 1μl of 20mg/ml BSA, DNA solution (up to 32.5μl containing 20-100ng of DNA). At this point, the mixture was heated to 37°C for 10 minutes to allow oligonucleotide primers to anneal to the DNA. Subsequently, 10-20μCi of α-[^{32}P]-dCTP and 2 units of Klenow enzyme were added. The mixture was incubated at room temperature for 2.5-18 hours, to allow polymerisation to occur, and the reaction terminated by the addition of 0.2ml of "oligo-stop solution" (20mM NaCl, 20mM Tris-HCl (pH7.5), 2mM EDTA, 0.25% SDS,

1mM dCTP). Incorporation was assayed by TCA precipitation and was usually in excess of 50%.

Oligo-labelling buffer was prepared by the mixing of three solutions, A:B:C, in the ratio 100:250:150 and could be stored at -20°C with freeze-thawing over many months.

Solution O: 1.25M Tris-HCl (pH8.0), 125mM $MgCl_2$; stored at 4°C.

Solution A: 1.0ml solution O + 18μl 2-mercaptoethanol + 5μl dATP + 5μl dGTP + 5μl dTTP (each at 0.1M in 3mM Tris-HCl (pH7.0), 0.2mM EDTA); stored at -20°C.

Solution B: 2M HEPES-Na^+ (pH6.6); stored at 4°C.

Solution C: Hexadeoxynucleotides dissolved in TE buffer at 90 $OD_{.260nm}$ units per ml; stored at -20°C.

2.4.17 Labelling of DNA Oligonucleotide Probes

Oligonucleotide DNA was 5'-end labelled for use as a probe by a slight modification of the method of Maxam and Gilbert (1980). The labelling reaction was performed in a total volume of 10μl containing 20 pmoles of oligonucleotide, 1μl of 10x kinase buffer (700mM Tris-HCl (pH7.6), 100mM $MgCl_2$, 1mM KCl, 50mM DTT; stored at -20°C), 10μCi of γ-[^{32}P]-ATP and at least 2 units of T4 polynucleotide kinase (in not more than 1μl). The kinase reaction was allowed to proceed for 30 minutes at 37°C and was terminated by dilution to 1ml with 10mM potassium phosphate (pH7.0). Unincorporated nucleotide was then removed by passage over Sephadex G-25 (NAP-10 column, Pharmacia) as follows; the column was pre-equilibrated with 4 bed volumes of 10mM potassium phosphate (pH7.0), the diluted labelling reaction (1ml) loaded and the oligonucleotide eluted with 1.5ml of the same buffer.

2.4.18 Southern Blotting from Agarose Gels

The transfer of DNA from agarose gels to nitrocellulose membranes was performed by the method of Southern (1975). The DNA sample was electrophoresed through an agarose gel and photographed as described in Section 2.4.6. After any unused parts of the gel were cut off, it was soaked in several volumes of Southern

denaturation buffer (1.5M NaCl, 0.5M NaOH) for 1 hour at room temperature with shaking to denature the DNA. The gel was neutralised by soaking in several volumes of Southern neutralisation buffer (1M Tris-HCl (pH8.0), 1.5M NaCl) for 1 hour at room temperature with shaking. A double sheet of Whatman 3MM paper (pre-wetted with 10xSSC buffer) was placed over a glass plate with the ends dipping into a reservoir filled with 10xSSC buffer and the gel was placed, with the original underside uppermost, on the damp 3MM paper. A piece of nitrocellulose membrane (Schleicher and Schuell BA85) was cut slightly larger than the gel, pre-wetted in 2xSSC buffer and placed over the gel. Two pieces of 3MM paper, pre-wetted in 2xSSC, buffer were placed over the nitrocellulose, then a 5-8cm stack of paper towels was placed on top. Finally a glass plate was placed on top of the stack and weighed down with a 500g weight. Transfer of DNA was allowed to proceed for 12-20 hours after which the nitrocellulose was removed, rinsed in 6xSSC for 5 minutes and baked for 2 hours at 80°C under vacuum. The membrane was used immediately for hybridisation experiments (see Section 2.4.20).

2.4.19 Colony Blotting

Colony screening to identify recombinant clones was performed by the method of Grunstein and Hogness (1975) as described by Maniatis et al. (1982). Transformed E.coli colonies were picked, using sterile toothpicks, and streaked in a grid pattern on duplicate LB-agar (plus ampicillin) plates and grown overnight. One of the duplicate plates was stored at 4°C as the master plate (colonies which carried the desired recombinant DNA sequences were later recovered from this) while the bacterial streaks on the other were transferred to a nitrocellulose membrane. This was achieved by gently laying the membrane (Schleicher and Schuell BA85) on top of the agar, causing the bacteria to stick to it. The membrane was then removed and laid, colony side uppermost, on Whatman 3MM paper soaked with Southern denaturation buffer (see Section 2.4.18) for 5 minutes. This step was repeated with 3MM paper soaked in Southern neutralisation buffer (see Section 2.4.18) for 5-10 minutes and 3MM paper soaked in 2xSSC. The membrane was air-dried, baked at 80°C under vacuum for 2 hours and used immediately for hybridisation experiments (see Section 2.4.20).

2.4.20 Hybridisation of Southern and Colony Blots

Nitrocellose membranes carrying DNA blotted from an agarose gel (Section 2.4.18) or DNA liberated from blotted bacterial colonies (Section 2.4.19) were hybridised with radiolabelled DNA probes synthesised as described in Section 2.4.16. The membrane was sealed in a plastic bag with 10-30ml of pre-hybridisation buffer (2xSSC, 10x Denhardt's solution [see below], 0.1mg/ml heat-denatured salmon-sperm DNA) and incubated at 65°C for at least 2 hours with constant shaking. The probe was denatured by heating to 100°C for 5 minutes and added to the bag and hybridisation was continued for 12-20 hours at 65°C with shaking. Probe which had bound to the membrane non-specifically was removed by a series of washes as follows; three 5 minute washes in 2xSSC at room temperature, two 30 minute washes in 2xSSC, 0.5% SDS at 65°C and two 10 minute washes in 0.1xSSC at room temperature. The membrane was blotted dry on Whatman 3MM paper, covered with Saran Wrap and autoradiographed.

Denhardt's solution was prepared at 50x concentration (1% (w/v) Ficoll, 1% (w/v) BSA, 10% (w/v) SDS, 1% (w/v) polyvinyl pyrrolidine; stored at room temperature).

2.4.21 Northern Blotting from Polyacrylamide Gels

Total yeast RNA or RNA recovered from an immunoprecipitation reaction (see Section 2.7.12) was electrophoresed through a denaturing polyacrylamide gel (Section 2.4.8) and transferred to a nylon membrane for hybridisation experiments. The gel plates were dismantled and the area of interest of the gel (estimated from the position of the marker dyes) was cut from the remainder, while still attached to one of the gel plates. The gel piece was transferred onto a piece of Whatman 3MM paper and this was placed, gel side uppermost, on several sheets of 3MM paper soaked in 0.5xTBE to wet the 3MM paper. Two Scotchbrite pads, three further pieces of 3MM paper and a piece of nylon membrane (Hybond N, Amersham) were also soaked in 0.5xTBE buffer. This was then assembled into the cassette of a Bio-Rad Trans-Blot electroblotting unit as follows; on top of one Scothbrite pad was placed one piece of 3MM paper, followed by the piece carrying the gel (gel side uppermost), the nylon membrane, the remaining two pieces of 3MM paper and, finally, the second Scotchbrite pad. Extreme care was taken at each stage to ensure that all air bubbles were excluded

from the assembly. The cassette was inserted in the Trans-Blot electroblotting tank (with the gel side oriented toward the cathode), the tank filled with 0.5xTBE buffer and transfer performed at 60V for 1-1.5 hours, with cooling supplied by a water-cooling system. After transfer, the cassette was disassembled and the membrane dried at 37°C for 10 minutes. The RNA was fixed on the membrane by wrapping in Saran Wrap and irradiating, RNA side down, on a UV transilluminator for 5 minutes. The membrane was used immediately in hybridisation experiments to detect snRNAs (see Section 2.4.22).

2.4.22 Hybridisation of Northern Blots

Hybridisation of Northern blots (Section 2.4.21) with oligonucleotide probes was performed by a method based on that of Church and Gilbert (1984). The membrane was re-wetted in dH_2O and sealed in a plastic bag with 10-20ml pre-hybridisation buffer (0.5M sodium phosphate (pH7.0), 7% SDS, 1mM EDTA). After incubation at 28°C for 1 hour, with constant agitation, the 5'-end labelled oligonucleotide probe (see Section 2.4.17) was added in the 1.5ml volume in which it was eluted from the NAP-10 column. Hybridisation was continued for 18-22 hours at 28°C with constant agitation, after which the membrane was washed in 0.5M sodium phosphate (pH7.0), 5% SDS, 1mM EDTA for 20 minutes at 28°C with shaking. The membrane was blotted dry and autoradiographed.

2.4.23 In Vitro Transcription Reaction

Radiolabelled RNA, for use in in vitro splicing reactions (see Section 2.5.2) and for probing protein blots (see Section 2.7.10), was synthesised in vitro from DNA cloned downstream from of their cognate promoters by SP6 (Melton et al., 1984) or T7 (Davanloo et al., 1984) RNA polymerases. The reaction was performed in 20μl volume containing the following; 0.2μg of linearised template DNA (there was no requirement to purify this from the restriction digestion reaction mix), 2μl 10x transcription buffer (400mM Tris-HCl (pH7.5), 60mM $MgCl_2$, 100mM DTT, 1mg/ml BSA; stored at -20°C), 10 units RNAsin (Promega), 25μM UTP, 0.5mM each of ATP, CTP and GTP, 20μCi of α-[^{32}P]-UTP, 2-5 units of the appropriate RNA polymerase. If the RNA was to be

capped, 2μl of 10mM G(5')ppp(5')G cap analogue (Pharmacia) was included in the reaction mix and the GTP concentration was reduced to 0.1mM. The polymerisation reaction was carried out at 37°C for 30-60 minutes and stopped on ice. Incorporation of radiolabel was generally in the range of 30-60%, depending on the particular template. Labelled RNA synthesised in this way was stored at -20°C for 1-2 weeks.

2.4.24 3'-End Labelling of RNA

RNA purified from an immunoprecipitation reaction was 3'-end labelled by the method of England et al. (1980). The dried RNA pellet from the ethanol precipitation was resuspended directly in 10μCi of cytidine 3',5'-[5'-^{32}P]-bisphosphate (1μl) and 2μl of reaction mix was added to give final conditions of; 50mM HEPES-NaOH (pH7.5), 10mM $MgCl_2$, 3mM DTT, 10% (v/v) deionised DMSO, 10μg/ml BSA, 25μM ATP, 5 units of RNAsin (Promega), 2 units T4 RNA ligase (BRL). The reaction was allowed to proceed for 16-20 hours at 4°C and was terminated by the addition of 57μl of 0.3M sodium acetate (pH5.2) and phenol/chloroform extraction. Labelled RNA was ethanol precipitated and analysed by electrophoresis through a 6% polyacrylamide gel (see Section 2.4.8) until the xylene cyanol dye had migrated approx. 30cm and autoradiography.

2.5 IN VITRO SPLICING

2.5.1 Preparation of Yeast Splicing Extract

Yeast whole cell extract competent for in vitro splicing was prepared by a method slightly modified from that of Lin et al. (1985). Cells of strain BJ2412 were grown overnight at 30°C in 500ml of YPDA medium to an OD_{650nm} of 0.4-0.5 and harvested by centrifugation in a Sorvall GS-3 rotor at 5,000rpm (4,200g) for 10 minutes at room temperature. The cells were resuspended in 50ml of 50mM potassium phosphate (pH7.5), transferred to a 50ml Falcon tube and spun down in a Centra-4X bench-top centrifuge at 3,000rpm (1,600g) for 5 minutes at room temperature. The cells were resuspended in 40ml of lyticase buffer (1.2M sorbitol, 50mM potassium phosphate (pH7.5), 30mM DTT) and 2,500 units of lyticase (Sigma; dissolved in H_2O

at 2,500 units/ml and stored at 4°C) were added. Cell wall digestion was allowed to proceed for 30 minutes at 30°C with gentle agitation and the spheroplasts thus generated were washed twice with 1.2M sorbitol by gentle resuspension and centrifugation as before. The spheroplasts were transferred to an Erlenmeyer flask with 200ml of YPDAS medium and incubated for 2 hours at 30°C with gentle agitation to allow metabolic recovery. The spheroplasts were harvested by centrifugation in a Sorvall GS-A rotor at 5,000rpm (4,000g) for 10 minutes at room temperature, resuspended in 50ml of SB buffer (1.2M sorbitol, 50mM Tris-HCl (pH7.5), 10mM $MgCl_2$, 3mM DTT) and transferred to a 50ml Falcon tube, then spun down in a bench-top centrifuge as before and weighed. All subsequent steps were performed in the cold-room at 4°C. For each gram of spheroplasts, 1ml of ice-cold buffer A (10mM HEPES-KOH (pH7.0), 10mM KCl, 1.5mM $MgCl_2$, 0.5mM DTT) was added, the spheroplasts resuspended and transferred to the receptacle of a 30ml Dounce tissue homogeniser and incubated on ice for 5 minutes. After this incubation to allow the spheroplasts to swell osmotically, they were lysed by 10-12 strokes of a tight-fitting Teflon pestle and the lysate transferred to a 10ml beaker on ice. Exactly 1/9th volume of 2M KCl was added drop-wise, with gentle stirring, to give a final concentration of 0.2M. The lysate was left, stirring gently on ice, for 30 minutes before being transferred to a 30ml polycarbonate tube and centrifuged in a Sorvall SS-34 rotor at 17,000rpm (35,000g) for 30 minutes at 4°C. The supernate was transferred to a 12ml screw-top polycarbonate tube (avoiding the floating, cloudy lipid layer) and centrifuged in a Beckman Ti50 rotor at 37,000rpm (95,000g) for 1 hour at 4°C. This high-speed supernate was collected (again avoiding any residual lipid) and dialysed against 2 changes of 1 litre of buffer D (50mM KCl, 20mM HEPES-KOH (pH7.0), 0.2mM EDTA, 0.5mM DTT, 20% (v/v) glycerol) for 3 hours at 4°C and any protein which precipitated during dialysis was cleared by centrifugation in a microfuge (13,800g) for 10 minutes at 4°C. The protein concentration of the extract was determined (see Section 2.7.1) and was usually in the range 10-25 mg/ml. The extract was snap-frozen in $N_2(l)$ in small aliquots and stored at -70°C.

2.5.2 Yeast In Vitro Splicing

Yeast in vitro splicing reactions were performed essentially as described by Lin et al. (1985). The components of the reaction, other than whole-cell extract, were mixed as follows; 1μl of a dilution of a T7 or SP6 transcription reaction (see Section 2.4.23) containing 20,000 dpm of uncapped, labelled substrate RNA, 1μl 30% (w/v) PEG 8000, 1μl 25mM $MgCl_2$, 0.6μl 1M potassium phosphate (pH7.5), 0.6μl 40mM ATP and 0.8μl "IgG buffer" (prepared by mixing 0.5M potassium phosphate (pH7.5): glycerol: 0.1M citric acid (pH3.0; adjusted with 0.1M sodium citrate) in the ratios 3:1:2 to give a final pH value of approx. 7.0). Then 5μl of splicing extract (thawed and allowed to warm to room temperature) was added. The final reaction therefore contained 10mM HEPES-KOH, 25mM KCl, 0.1mM EDTA, 0.25mM DTT, 11.33% (v/v) glycerol, 3% (w/v) PEG 8000, 2.5mM $MgCl_2$, 80mM potassium phosphate, 2.4mM ATP, 2.64mM citric acid and was allowed to proceed at 24°C for 2-30 minutes. After the reaction was stopped by the addition of 3μl of stop solution (1mg/ml proteinase K, 50mM EDTA, 1% SDS) and incubation at 37°C for 15 minutes, 0.2ml of splicing cocktail (50mM sodium acetate (pH5.3), 1mM EDTA, 0.1% SDS, 25 μg/ml tRNA) was added, the mixture phenol/chloroform extracted and the RNA ethanol precipitated. The pellet was redissolved in 1μl of ddH_2O and 2μl of urea load buffer was added before heating to 90°C for 2 minutes to denature the RNA and electrophoresing through a 6% polyacrylamide/7Murea gel (see Section 2.4.8). When the xylene cyanol dye had migrated approx. 30cm, electrophoresis was halted and the gel plates separated. The gel was transferred to Whatman 3MM paper, covered with Saran Wrap and subjected to autoradiography.

2.5.3 In Vitro Splicing in a HeLa Cell Nuclear Extract

In vitro splicing reactions in HeLa cell nuclear extracts were performed by a slight modification of the method described by Krainer et al. (1984). The components, other than the nuclear extract, were mixed at room temperature as follows; 1μl of a dilution of a SP6 transcription reaction (Section 2.4.23) containing 20,000 dpm of capped, labelled substrate RNA, 2.5μl 200mM KCl, 2μl 40mM $MgCl_2$, 2μl 0.1M creatine phosphate (Tris salt), 0.4μl 0.1M ATP and 7.1μl of ddH_2O. Then 10μl of nuclear

extract (which had been thawed and allowed to warm to room temperature) was added. The final reaction therefore contained 8mM HEPES-KOH (pH7.6), 60mM KCl, 3.2mM $MgCl_2$, 8mM creatine phosphate, 1.6mM ATP, 8% (v/v) glycerol, 0.08mM EDTA, 0.2mM PMSF. The was reaction allowed to proceed at 30°C for 0.3-3 hours before being stopped by the addition of 75μl of ddH_2O and 0.1ml of 2xPK buffer (0.2mM Tris-HCl (pH7.5), 25mM EDTA, 0.3M NaCl, 2% SDS, 4mg/ml proteinase K) and further incubation at 30°C for 30 minutes. The mixture was subjected to two rounds of phenol/chloroform extraction and the RNA was ethanol precipitated. The pellet was redissolved in 1μl of ddH_2O, 2μl of urea load buffer was added and the RNA denatured by heating to 90°C for 2 minutes. The RNA was electrophoresed through a denaturing 10% polyacrylamide/7M urea gel (see Section 2.4.8) at 30W until the xylene cyanol dye had migrated approx. 30cm and the gel was processed for autoradiography as for yeast splicing gels (Section 2.5.2).

2.6 DNA SEQUENCING

2.6.1 Preparation of Single-Stranded Phagemid DNA

An overnight culture of cells containing phagemid DNA was prepared by picking an individual ampicillin colony into 2ml of LB/ampicillin medium and incubating at 37°C and 40μl of this was used to inoculate 2ml of LB medium. This was incubated at 37°C with vigorous shaking for 30 minutes before being infected with helper phage M13KO7 at an m.o.i. of 20 (i.e. there are approx. $4x10^7$ bacteria present in the culture, so $8x10^8$ phage particles were added). The infected culture was shaken vigorously for a further 6-8 hours at 37°C before the cells were spun out by centrifugation in a microfuge (13,800g) for 15 minutes at room temperature and 1ml of the supernate transferred to a fresh 1.5ml Eppendorf tube. Secreted phage particles were precipitated by the addition of 0.25ml of ice-cold 20% (w/v) PEG 6000, 3.75M ammonium acetate, incubation on ice for 30 minutes and centrifugation in a microfuge (13,800g) for 15 minutes at room temperature. All traces of the supernate were removed, the pellet resuspended in 0.4ml TE buffer and chloroform extracted (with vortexing for a full minute). The aqueous phase was subjected to two phenol/chloroform extractions and one further chloroform extraction before 0.5 volumes of 7.5M ammonium acetate and

2 volumes of ethanol were added. The mixture was incubated at -20°C for 20 minutes to allow precipitation, and the phagemid DNA was pelleted by centrifugation in a microfuge (13,800g) for 15 minutes at 4°C. After a 70% ethanol wash and drying, the pellet was dissolved in 20μl of ddH_2O and stored at -20°C. A small aliquot of the preparation was analysed on an agarose gel (Section 2.4.6) when single-stranded phagemid DNA was clearly visible in greater abundance than M13KO7 helper phage DNA (8.7kb).

2.6.2 Sequencing Reactions

Sequencing of DNA was performed by the dideoxy-chain terminator method as described by Sanger et al. (1977). To 8μl of template DNA preparation (see Section 2.6.1) was added 2μl of annealing mix (freshly prepared by mixing 0.1M Tris-HCl (pH7.5), 0.1M $MgCl_2$ and 4μg/ml universal sequencing primer in the ratio 3:2). The mixture was heated to 85°C for 10 minutes and allowed to cool at room temperature for a further 10 minutes to permit annealing of the primer to the template DNA. Meanwhile, 2.5μl aliquots of sequencing solutions A, C, G and T (see Table 2.5) were dispensed into Eppendorf tubes. To the annealed template-primer mix, 8μCi of α-[^{35}S]-dATP and 2.5 units of Klenow were added and mixed gently and then 2.5μl aliquots of this were transferred to the side-walls of the tubes containing the A, C, G and T solutions. The tubes were spun in a microfuge for 1 second to mix the contents and thus start the reaction. After 15 minutes incubation at room temperature, 2.5μl of chase solution (see Table 2.5) was added to each of the four tubes and incubation continued at room temperature for a further 15 minutes. The reaction was terminated by the addition of 3μl of formamide load dye (deionised formamide, 20mM EDTA, 0.3% (w/v) xylene cyanol, 0.3% (w/v) bromophenol blue; stored at -20°C) and heating to 100°C for 2-5 minutes and was subsequently electrophoresed through a 6% polyacrylamide/7M urea gel as described in Section 2.4.8 (except that a BRL 5.7mm-Sharkstooth Comb was used instead of a conventional well comb) until the bromophenol dye had migrated approx. 40cm. Following electrophoresis, the gel plates were separated and the gel (still attached to one of the plates) immersed in 2 litres of fixing solution (10% (v/v) acetic acid) for 15 minutes. The gel was then removed from the fixing solution, thoroughly drained and transferred from the glass plate to a piece

of Whatman 3MM paper (achieved by pressing the 3MM paper firmly onto the gel and lifting carefully off). The gel was covered with Saran Wrap, dried under vacuum at 80°C for 2 hours and autoradiographed (after removal of the Saran Wrap).

TABLE 2.5 Composition of Sequencing Solutions

(all concentrations in μM)

	Solution				
	A	C	G	T	chase
dATP	-	-	-	-	1000
dCTP	110	4	82	82	1000
dGTP	110	82	4	82	1000
dTTP	110	82	82	4	1000
ddATP	33.3	-	-	-	-
ddCTP	-	67.5	-	-	-
ddGTP	-	-	150	-	-
ddTTP	-	-	-	250	-

(all solutions in TE buffer and stored at -70°C)

2.7 PROTEIN METHODS

2.7.1 Quantitation of Proteins

Dilutions of the test sample were prepared and 2μl of each were spotted onto a piece of Whatman No.1 filter paper alongside 2μl aliquots of protein concentration standards (BSA, 50-1000μg/ml; stored at -20°C) and dried at 65°C for a few minutes. The filter was then incubated in ESAU stain (25% (v/v) propan-2-ol, 10% (v/v) acetic acid, 1% (w/v) Coomassie Brilliant Blue (R250); stored at room temperature and can be re-used many times) for 15 minutes at room temperature and destained with tapwater until the background staining was low. The protein concentration in the test sample was then estimated by comparison with the standards of known concentration.

2.7.2 SDS-Polyacrylamide Gel Electrophoresis of Proteins

TABLE 2.6 Composition of SDS-Polyacrylamide Gel Mixes

Stock Solns.	8.5% Separating Gel	5% Stacking Gel
30% (w/v) acrylamide*	8.5ml	1.67ml
1.25% (w/v) bisacrylamide*	4.0ml	1.3ml
1M Tris-HCl (pH8.8)	11.2ml	-
1M Tris-HCl (pH6.8)	-	1.25ml
dH_2O	6.2ml	5.78ml
20% (w/v) SDS	0.15ml	50μl
10% (w/v) ammonium persulphate	0.15ml	100μl
TEMED	15μl	10μl

(*Stock acrylamide and bisacrylamide solutions were deionised, filtered and stored at 4°C)

Proteins were separated by SDS-discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). An 8.5% separating gel mix (Table 2.6) was prepared and poured between two 16x16cm glass plates (separated at their edges by 1.5mm spacers, sealed with a piece of rubber tubing and clamped into an ATTO gel-casting apparatus, model SJ-1060) to within 5cm of the top of the notched plate. Water was gently layered over the gel mix and the assembly left in an upright position at room temperature for 1 hour to allow polymerisation. The water was poured off the surface, stacking gel mix (Table 2.6) was poured onto the separating gel up to the top of the notched plate and a Teflon slot-forming comb (12x0.5cm wells or 20x0.3cm wells) was inserted. After at least 30 minutes, the stacking gel was fully polymerised and the comb and sealing rubber tubing were removed. The gel assembly was transferred to an ATTO electrophoresis tank (model SJ-1060) which was filled with SDS-PAGE buffer (25mM Tris- base, 192mM glycine, 1% (w/v) SDS (pH8.8); prepared as a 10x stock). Samples were prepared by mixing with an equal volme of 2x protein load buffer (125mM Tris-HCl (pH6.8), 200mM DTT, 4% (w/v) SDS, 40% (v/v) glycerol, 0.02% (w/v) bromophenol blue; stored at 4°C for up to 3 weeks) and heating to 100°C

for 5 minutes before being loaded on the gel. Electrophoresis was performed at 50-200V for 3-16 hours. Size markers in the range 29-205kD were from Sigma.

2.7.3 Staining of Protein Gels

2.7.3.1 Coomassie Staining

After SDS-PAGE (see Section 2.7.2) was completed, the gel plates were separated and the gel transferred to a plastic container where it was incubated at 37°C for 30-60 minutes in sufficient staining solution (25% (v/v) propan-2-ol, 10% (v/v) acetic acid, 0.05% (w/v) bromophenol blue) to submerge the gel. The staining solution was then removed, the gel washed briefly with water and 100-200ml of destaining solution (25% (v/v) methanol, 10% (v/v) acetic acid) added. The gel was destained at 37°C until the background was low, i.e. 2-16 hours. For storage the gel was transferred onto a sheet of Whatman 3MM paper, covered with Saran Wrap and dried for 2 hours at 80°C on a vacuum gel drier.

2.7.3.2 Silver Staining

Detection of very small amounts of protein in SDS-polyacrylamide gels was achieved by the method of Morrisey (1981). Following SDS-PAGE, the gel was transferred to a plastic container (kept exclusively for this purpose) and incubated at room temperature with constant agitation in the following: 100ml 50% (v/v) methanol, 10% (v/v) acetic acid for 30 minutes; 100ml 5% (v/v) methanol, 7% (v/v) acetic acid for 30 minutes; 50ml 10% (v/v) glutaraldehyde for 30 minutes; several changes of 100ml dH_2O over 2 hours; 5μg/ml DTT for 30 minutes; 0.1% (w/v) silver nitrate for 30 minutes. The gel was then rinsed twice rapidly in dH_2O and once rapidly in developer solution (3% (w/v) sodium carbonate, 0.02% (v/v) formaldehyde) before being incubated in 100ml of developer solution until staining reached the desired intensity. Development was halted by the addition of 5ml of 2.3M citric acid and incubation for 10 minutes and the gel was then rinsed in several changes of 100ml dH_2O over 30 minutes. Finally the gel was incubated in 100ml 0.03% (w/v) sodium carbonate for 30 minutes and was stored sealed in a plastic bag at 4°C. It was possible to silver stain a gel which had

previously been Coomassie stained with the omission of the first step, i.e. incubation in 50% (v/v) methanol, 10% (v/v) acetic acid.

2.7.4 Extraction of Total Cellular Protein for Electrophoresis

2.7.4.1 Escherichia coli Protein

An overnight culture of E.coli cells was diluted 1:100 into fresh medium and grown to the desired cell density with induction of fusion protein expression as required (see Section 2.7.5). The culture was cooled on ice for 15 minutes and the cells from 1ml were harvested by centrifugation in the bench-top Centra-4X centrifuge at 3,000rpm (1,600g) for 5 minutes. The cells were resuspended in 50μl of dH_2O, by vortexing, 50μl of 2x protein load buffer (see Section 2.7.2) was added and the cells lysed by heating to 100°C for 5 minutes. The lysed culture was centrifuged in a microfuge (13,800g) for 5 minutes to clear insoluble material and 15-20μl of the supernate was loaded on a 0.5cm well for SDS-PAGE. The remainder of the supernate was snap-frozen in N_2(l) and stored at -70°C if necessary.

2.7.4.2 Yeast Protein

A 100ml yeast cell culture (in YPDA or supplemented YMM medium as appropriate) was grown overnight to late logarithmic phase, i.e. until the OD_{650nm} was in the range 0.6-1.0, transferred to 50ml Falcon tubes and the cells harvested by centrifugation in a Centra-4X centrifuge at 3,000rpm (1,600g) for 10 minutes at room temperature. The cells were washed by resuspension in 50ml 1xPBS buffer and centrifugation as before and were then resuspended in 2ml of 1xPBS. The cell suspension was transferred to microfuge tubes and the cells spun down by centrifugation in a microfuge (13,800g) for 30 seconds at room temperature. To each cell pellet was added an equal volume of sonication buffer (1xPBS, 0.1% NP40, 1mM PMSF, 1mM DTT and (optionally) 5μg/ml each of pepstatin A and leupeptin; freshly prepared) and each was sonicated at full power for four bursts of 30 seconds each (with 2 minutes on ice between bursts). The lysate was centrifuged in a microfuge (13,800g) for 2 minutes at 4°C and the supernate was collected and centrifuged in a microfuge (13,800g) for 15

minutes at 4°C. An equal voume of 2x protein load buffer (see Section 2.7.2) was added to the supernate and the protein denatured by heating to 100°C for 5 minutes. Aliquots of this extract (5-25μl) were subjected to SDS-PAGE while the remainder was snap-frozen in N_2(l) and stored at -70°C. Alternatively the supernate from the second spin was used directly for immunoprecipitation reactions (see Section 2.7.12) without denaturation in protein load buffer.

2.7.4.3 Drosophila melanogaster Protein

The cells from a culture of Drosophila melanogaster cells were resuspended in 5 volumes of 1x protein load buffer (see Section 2.7.2) and heated to 100°C for 5 minutes before being centrifuged in a microfuge (13,800g) for 2 minutes at room temperature. Aliquots of the supernate (10-15μl) were subjected to SDS-PAGE while the remainder was snap-frozen in N_2(l) and stored at -70°C.

2.7.5 Induction of Fusion Protein Synthesis in E.coli

2.7.5.1 pUR Vector System

E. coli BMH71-18 or NM522 cells carrying pUR construct or parent vector plasmid were grown overnight at 37°C in 5ml of LB/ampicillin medium and and this was diluted 1:100 into fresh LB/ampicillin medium (5ml for analytical purposes (Section 2.7.4.1) and 200ml for preparative purposes (Section 2.7.6)). The culture was incubated at 37°C with shaking until the $OD_{.650nm}$ reached 0.4 and then 0.004 volume of 24mg/ml IPTG was added and the incubation continued for a further 1 hour.

2.7.5.2 pEX Vector System

E. coli pop2136 cells carrying pEX construct or parent vector plasmid were grown overnight in 5ml LB/ampicillin medium at 30°C and this was diluted 1:100 into fresh LB/ampicillin medium (volumes as in Section 2.7.5.1). The culture was incubated at 30°C with shaking until the $OD_{.650nm}$ reached 0.4, when it was shifted to a water

bath at 42°C for a few minutes. Incubation was continued at 42°C with vigorous shaking for 1 hour.

2.7.5.3 pATH Vector System

Induction of trpE fusion polypeptide synthesis in E. coli HB101 cells carrying pATH construct or parent vector plasmid was performed by the method of Spindler et al. (1984). Cells were grown overnight in 5ml M9+CA+W/ampicillin medium at 37°C and this was diluted 1:10 into M9+CA/ampicillin (volumes as in Section 2.7.5.1). This culture was incubated at 37°C with vigorous shaking for 1 hour before 0.005 volume of 1mg/ml indoleacrylic acid (in ethanol; stored at -20°C) was added and incubation continued for a further 2 hours with vigorous shaking at 37°C.

2.7.6 Purification of Fusion Proteins from E.coli

2.7.6.1 Purification by SDS-PAGE

A 200ml culture was induced for fusion protein synthesis (see Section 2.7.5) and a 15ml crude SDS-lysate was prepared as described in Section 2.7.4.1. A preparative 8.5% SDS-polyacrylamide gel was poured as described in Section 2.7.2 except that, instead of inserting a Teflon comb to form individual wells, dH_2O was layered on top of the stacking gel to allow it to set level, effectively forming one large well spanning the entire width of the gel. Crude SDS-lysate (0.5-1.0ml per gel) was electrophoresed through the gel and vertical reference strips (0.3-0.5mm wide) were cut from either edge and from the middle of the gel and Coomassie stained (Section 2.7.3.1). By lining up these strips with the remainder of the gel it was possible to excise the horizontal strip of gel containing the fusion protein. The strips were placed in a section of dialysis tubing which was then filled with 3-5ml SDS-PAGE buffer (Section 2.7.2) and clipped at either end to form a bag. This assembly was placed in a BRL horizontal gel electrophoresis tank and submerged in SDS-PAGE buffer and the protein was eluted from the gel pieces at 150V for 4 hours. The current was reversed for 15 seconds to detach the eluted protein from the dialysis tubing and the liquid was recovered from the bag. The fusion protein solution was concentrated by centrifugation in a MSE Minor

'S' fixed-angle rotor bench-top centrifuge at 3,000rpm (1,000g) in a Centricon 30 microconcentrator (Amicon Corporation) at 4°C to a volume of 1ml. An aliquot of purified protein was subjected to SDS-PAGE (Section 2.7.2) to assess the recovery, which was generally in the range 50-100μg per gel. The protein was stored frozen at -70°C.

2.7.6.2 Purification in Insoluble Protein Fraction

A 200ml E. coli culture was induced for fusion protein synthesis (see Section 2.7.5) and the insoluble protein fraction prepared by a modification of the method of Hall et al. (1984). Following induction, the cells are harvested by centrifugation in a Sorvall GS-A rotor at 3,000rpm (1,500g) for 5 minutes at 4°C, resuspended in 4ml of 15% (w/v) sucrose, 50mM Tris-HCl (pH8.0), 50mM EDTA, and transferred to a 30ml polycarbonate tube before 0.4ml of 10mg/ml lysozyme was added. After thorough mixing the tube was left on ice for 40 minutes, with intermittent gentle agitation, and 5.6ml of 0.2% (v/v) Triton X-100 in TE buffer was added. After 5 minutes incubation on ice, the mixture was spun in a Sorvall SS-34 rotor at 19,000rpm (43,000g) for 10 minutes at 4°C and the supernate was discarded. The insoluble protein pellet was either resuspended in 1x protein load buffer (see Section 2.7.2) and heated to 100°C for 5 minutes, for SDS-PAGE, or was solubilised to give renatured protein as follows; 2-5ml of 6M urea was added, the pellet sonicated at full power for 3x15 seconds on ice and the mixture recentrifuged in a Sorvall SS-34 rotor as above. The supernate was dialysed against three changes of 1 litre each of 1xPBS buffer at 4°C. The protein preparation was concentrated in a Centricon 30 microconcentrator (see Section 2.7.6.1) and stored at -70°C.

2.7.7 Immunisation of Rabbits

All experiments on live animals were performed in compliance with the Cruelty to Animals Act (1986) under licence number 63181. Rabbits were females of the half-lop variety and were obtained from Froxfield Rabbits, Hampshire.

The volume of fusion protein preparation (see Section 2.7.6) containing the required amount (50-100μg for the first injection and 20-50μg for all subsequent

injections) was diluted to 1ml with dH_2O. To this was added 1ml of Freund's complete adjuvant (for the first injection) or Freund's incomplete adjuvant (for all subsequent injections), the mixture was vortexed until a stable emulsion had been formed and this was injected into the rabbit at several subcutaneous sites using a 25-guage needle. Immunisation was repeated at monthly intervals.

Blood was collected from the ear vein (10ml) prior to the first injection (pre-immune) and 10-12 days after the second and all subsequent injections (immune). Ten days after the final injection the rabbit was anaesthetised with Sagatal (May and Baker Ltd., Dagenham), blood (75-125ml) was withdrawn directly from the heart, using a 19-guage needle and syringe, and the rabbit was finally killed with a further injection of Sagatal.

2.7.8 Serum Preparation and Storage

Blood collected as described (see Section 2.7.7) was allowed to clot for 1 hour at room temperature and the clot freed from the walls of the container using a glass rod. The blood was left at 4°C overnight to allow the clot to retract, the serum was withdrawn to a 10ml or 50ml Falcon tube and centrifuged in a bench-top centrifuge at 3,000rpm (1,600g) for 2 minutes to remove red cells. Some (or all, for smaller bleeds) of the serum was aliquoted into Eppendorf tubes and snap-frozen in $N_2(l)$ for storage at -70°C, while the remainder was snap-frozen in the Falcon tube and stored at -70°C. When serum was required, an aliquot was thawed on ice, the desired volume withdrawn and the remainder immediately snap-frozen as above. Each individual aliquot was subjected to no more than six freeze-thaw cycles.

2.7.9 Western Blotting

Electrophoretic transfer of proteins from SDS-polyacrylamide gels to nitrocellulose membrane was performed by a modification of the method of Towbin et al. (1979). Following SDS-PAGE (see Section 2.7.2), the gel and a piece of nitrocellulose membrane, cut to the same size as the gel, were soaked in 1x Western transfer buffer (20mM Tris base, 150mM glycine) for 30 minutes at room temperature. Two Scotchbrite pads and four pieces of Whatman 3MM paper, cut to the size of the

Scotchbrite pads, were wetted in the same buffer and the following was assembled in the cassette of a BioRad TransBlot electroblotting unit; on top of the first Scotchbrite pad was placed two pieces of 3MM paper, then the gel, the nitrocellulose membrane, the other two pieces of 3MM paper and finally the second Scotchbrite pad. The cassette was inserted (gel oriented toward the anode) into the TransBlot electroblotting tank which was filled with 1x Western transfer buffer. Proteins were transferred to the membrane at 65V for 3 hours or at 25V for 16 hours, with cooling supplied by a water-cooling system. Following transfer, the nitrocellulose membrane was dried at 37°C for 15 minutes, re-wetted in dH_2O, stained for 2 minutes in Ponceau S (Sigma) and destained by washing with dH_2O several times. This allowed visualisation of the proteins on the membrane and, therefore, the transfer could be verified and the membrane cut to strips if required. The membrane was incubated in 20-30ml blocking solution (see Table 2.7) for 2-4 hours at 37°C with agitation and then incubated for 16-20 hours at 4°C with agitation in primary antibody diluted (1:500-1:2000 of polyclonal serum) in the appropriate antibody solution (Table 2.7, approx. 1ml per $10cm^2$ of membrane). The membrane was washed in 5 changes of wash buffer (50ml 1xTBS, 0.1% (v/v) NP40) for 5 minutes each at room temperature with shaking and then incubated at room temperature for 1 hour, with agitation, in 10ml of a dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Promega Biotec, 1:7,500 in the appropriate antibody solution; see Table 2.7). The membrane was then washed as above and was placed in 5-10ml of freshly prepared development solution (0.1M Tris-HCl (pH9.5), 0.15M NaCl, 5mM $MgCl_2$, 0.33mg/ml BCIP*, 0.165mg/ml NBT*; *both supplied at 50mg/ml in the Promega development substrate kit) until the staining of the bands reached the desired intensity. Development was terminated by washing the membrane in an excess of dH_2O and the membrane was blotted dry, covered with Saran Wrap and stored in the dark at room temperature.

TABLE 2.7 Composition of Immunoblotting Solutions

	Blocking Solution	Antibody Solution
I.	5% (w/v) BSA, 1xTBS, 0.1% (w/v) NaN_3	1% (w/v) BSA, 1xTBS, 0.1% (w/v) NaN_3
II.	1xTBS, 0.2% (v/v) Tween 20	as blocking solution
III.	1xTBS, 1% (w/v) ovalbumin, 0.1% (w/v) NaN_3	as blocking solution

(all solutions freshly prepared)

2.7.10 North-Western Blotting

Proteins fractionated by SDS-PAGE and transferred to a nitrocellulose filter were probed for RNA binding properties by the method of Gerke and Steitz (1986). Western blotting was performed as described in Section 2.7.9 but, after the Ponceau S staining step, the membrane was incubated for at least 1 hour with several changes of 50ml binding buffer (10mM Tris-HCl (pH7.5), 50mM NaCl, 1mM EDTA, 0.02% (w/v) BSA, 0.02% (w/v) polyvinyl pyrrolidine, 0.02% (w/v) Ficoll; freshly prepared) at room temperature with agitation. The membrane was then sealed in a plastic bag and incubated in binding buffer (2ml per $10cm^2$) containing 20,000 dpm/ml of [^{32}P]-labelled RNA probe (from an in vitro transcription reaction; see Section 2.4.23) and 10μg/ml tRNA for 1 hour at room temperature with shaking. After binding, the membrane was washed with 2-4 changes of 50ml binding buffer, for 5 minutes each, at room temperature with agitation, blotted dry, covered with Saran Wrap and autoradiographed.

2.7.11 Affinity Purification of Antibodies

Antibodies were purified from crude serum using antigen immobilised on nitrocellulose membrane by the method of Robinson et al. (1988). Preparative SDS-PAGE of a crude lysate of E. coli cells induced for fusion protein synthesis was performed as described in Section 2.7.6.1 and protein was Western blotted to a

nitrocellulose membrane as described in Section 2.7.9. The membrane was Ponceau S stained to visualise the protein pattern and the horizontal strip containing the fusion protein was excised and blocked as in Section 2.7.9 (blocking solution III). The membrane strip was then incubated with 10ml of the blocking solution containing a 1:100 to 1:300 dilution of polyclonal serum for 2-3 hours at room temperature or overnight at 4°C with agitation. The membrane was washed, as described in Section 2.7.9, and the bound antibodies were eluted by incubation in 2ml of 0.1M glycine-HCl (pH2.5), 0.1% (w/v) ovalbumin for 5 minutes followed by a second 2ml of the same buffer for 10 minutes, both at room temperature. Each batch of eluant was immediately neutralised by the addition of 0.5ml 1M Tris-HCl (pH7.5), the two batches were pooled and dialysed against three changes of 1 litre each of 1xTBS at 4°C over 16-20 hours. The affinity purified antibody solution was stored at 4°C in the presence of 0.1% (w/v) NaN_3.

2.7.12 Immunoprecipitation Reactions

2.7.12.1 High Stringency Immunoprecipitation from Labelled Extracts

In vivo labelling of yeast proteins, preparation of extracts and immuno-precipitation reactions were performed as described by Lee *et al.* (1986).

In vivo labelling of proteins was achieved by adding 0.5mCi of L-[^{35}S]-methionine to 400ml of supplemented YMM medium containing approx. $5x10^6$ yeast cells/ml and continuing incubation until the cell density was approx. $1x10^8$ cells/ml. Yeast total cell protein was extracted as described in Section 2.7.4.2, except that following the second clearing spin in the microfuge, 7 volumes of immunoprecipitation buffer (1xPBS, 1% (v/v) NP40, 10% (v/v) foetal calf serum, 1mM PMSF) was added.

For immunoprecipitation reactions, the diluted [^{35}S]-labelled extract was divided into 6-12 Eppendorf tubes (as required), to each aliquot was added 10μl of the appropriate polyclonal serum and the tube incubated at 4°C with mixing for 3-16 hours to allow antibody-antigen binding. To this was added a 1:1 slurry of swollen Protein A-Sepharose beads:NET buffer (50mM Tris-HCl (pH7.5), 0.15M NaCl, 5mM EDTA) and incubation continued for a further 1 hour to allow the immune-complexes to bind to the beads. The beads were then washed as follows (all at room temperature, each

wash being terminated by spinning out the beads in a microfuge (13,800g) for 30 seconds); 2x30 minutes in HEN buffer (0.1M HEPES-NaOH (pH7.9), 1M NaCl, 1% (v/v) NP40, 10mM EDTA, 1mM PMSF), 2x30 minutes in LIT buffer (0.5M LiCl, 0.1M Tris-HCl (pH8.8), 0.1% (w/v) SDS, 1% (v/v) NP40), and 10 minutes in HEN buffer again, all with constant mixing. After the final wash the beads were resuspended in 35μl of 1.33x protein load buffer (see Section 2.7.2), heated to 100°C for 5 minutes, spun in a microfuge (13,800g) for 2 minutes and the supernate electrophoresed through an 8.5% SDS-polyacrylamide gel (see Section 2.7.2). The gel was Coomassie stained (see Section 2.7.3.1), incubated in 50ml Amplify (Amersham) for 30 minutes at room temperature with agitation, covered with Saran Wrap and dried in a vacuum drier at 80°C for 2 hours. Immunoprecipitated proteins were visualised by autoradiography after removal of the Saran Wrap.

2.7.12.2 Low Stringency Immunoprecipitation from Yeast Extracts

Immunoprecipitation from protein extracts under conditions of low stringency was performed by a procedure developed in this laboratory by M. Lossky and described in Lossky et al. (1987).

Protein A-Sepharose (PAS) beads were swollen by incubation in NTN buffer (50mM Tris-HCl (pH7.5), 0.15M NaCl, 0.1% (v/v) NP40) for 10 minutes and were then washed 4 times in the same buffer (these, and all subsequent washes, were performed by resuspending the beads in 1ml of buffer and then recovering them by centrifugation in a microfuge (13,800g) for 15 seconds). For each reaction, 20μl of swollen PAS was incubated with 3-15μl of the appropriate serum or 15μg of anti-m^{3}G antibody for 1 hour in 0.1ml total volume of NTN buffer with mixing. The PAS-IgG complexes formed were spun down and washed 4-5 times with 1ml of NTN buffer. For controls, IgG was adsorbed to PAS beads in the presence of purified fusion proteins (see Section 2.7.6), at a concentration of 1ug per 1ul of serum used, to form PAS-IgG-fusion protein complexes and thus block antigen binding sites on the IgG.

Splicing extract (see Section 2.5.1) or yeast total cell protein extract (see Section 2.7.4.2) was diluted with an equal volume of buffer such that final conditions were 10mM HEPES-KOH (pH7.0), 150mM Na^+/K^+ ions, 2.5mM $MgCl_2$, 0.1% (v/v) NP40 amd then added to the beads. Alternatively, splicing extract was assembled into a

mock-splicing reaction (as described in Section 2.5.2, but with no RNA substrate added, -/+ ATP), then diluted with an equal volume of buffer, such that the final conditions were as above, and added to the beads. The tube was incubated at 4°C for 2 hours, with mixing, to allow the IgG to bind its antigen and the beads were washed 4-5 times with NTN buffer and once with NT buffer (50mM Tris-HCl (pH7.5), 0.15M NaCl). At this point the beads were treated for SDS-PAGE as described in Section 2.7.12.1, electrophoresed and Western blotted (see Section 2.7.9) or the precipitated RNA was purified for analysis as described below.

The beads were incubated in 50μl of proteinase K solution (50mM Tris-HCl (pH7.5), 0.3M NaCl, 5mM EDTA, 1.5% (w/v) SDS, 2mg/ml proteinase K; stored at -20°C) for 30 minutes at 37°C and then 50μl dH_2O was added. The mixture was extracted 3 times with phenol/chloroform, to release the RNA and remove the beads and protein, and ethanol precipitated (0.2-0.5μg of sonicated salmon sperm DNA, extensively deproteinised and treated overnight at room temperature with 0.3M NaOH, was added as carrier). The purified RNA was then 3'-end labelled and analysed directly by polyacrylamide gel electrophoresis (as described in Section 2.4.23) or was electrophoresed through a 6% polyacrylamide gel (Section 2.4.8) for Northern blot analysis (as described in Sections 2.4.21 and 2.4.22).

2.7.12.3 Low Stringency Immunoprecipitations from HeLa Cell Nuclear Extracts

Immunoprecipitation reactions from mock-splicing reactions in HeLa cell nuclear extracts (see Section 2.5.3), were performed as described for yeast extracts (Section 2.7.12.2), except that, in place of NTN and NT buffers, all washes were with HKMN buffer (20mM HEPES-KOH (pH7.6), 60mM KCl, 3.2mM $MgCl_2$, 0.1% (v/v) NP40) and the mock-splicing reaction mix was not diluted prior to incubation with the PAS-IgG.

2.7.13 Non-denaturing Gel Electrophoresis

Non-denaturing gel electrophoresis of yeast cell extracts was performed by the method of Pikielny et al. (1986). A vertical, 15cmx15cmx1.5mm gel was cast from 3% (w/v) acrylamide (60:1, acrylamide : bisacrylamide), 0.5% (w/v) agarose, 0.5X TBE. To each sample was added 1/5th volume of load buffer (50% (v/v) glycerol, 2.5xTBE, 0.05%

(w/v) each xylene cyanol and bromophenol blue; stored at -20°C), the mix was loaded on 1cm wells in the gel and electrophoresis was carried out in 0.5xTBE at 150V for approximately 3 hours at 4°C.

For Northern hybridisation analysis, the fractionated sample was transferred from the gel to Hybond N membrane by electroblotting in 0.5xTBE at 60V and probed exactly as described for denaturing polyacrylamide gels (Sections 2.4.21 and 2.4.22).

Non-denaturing gel electrophoresis of HeLa cell extracts was performed as described by Konarska and Sharp (1987). This is essentially as described above for yeast extracts, except; (i) a 4% (w/v) acrylamide (80:1 acrylamide : bisacrylamide), 50mM Tris-glycine (50mM Tris base, 50mM glycine: pH8.8) gel was cast and run in Tris-glycine buffer, (ii) 1/10th volume load buffer (97% (v/v) glycerol, 1% (w/v) each xylene cyanol and bromophenol blue) was added to the sample, and (iii) the gel was pre-run for 15-30 minutes. For Northern hybridisation analysis, this was electroblotted to GeneScreen in running buffer at 0.2A for 16 hours at 4°C and treated as described (Sections 2.4.21 and 2.4.22).

CHAPTER 3

RAISING ANTISERA TO THE PRP8 PROTEIN

3.1 INTRODUCTION

Following the cloning of the PRP8 gene in this laboratory, an immunological approach was employed to identify the putative protein product and to commence analysis of its function (S.Jackson, in Jackson et al., 1988). Two regions from the centre of the PRP8 gene (8.1 and 8.2, see Figure 3.1A) were cloned into the 3' end of the E.coli lacZ gene of the pUR series of vectors to allow synthesis of β-galactosidase-PRP8 fusion proteins (FP8.1 and FP8.2) and these were used to raise antisera in rabbits (S.Jackson, in Jackson et al., 1988). The fusion constructs were made before the sequence of the PRP8 gene had been determined, and was achieved by cloning each fragment into the lacZ gene in all three reading frames and screening for fusion protein synthesis.

At the outset of this project, the sequence of almost the entire gene had been determined (S.Jackson; Ph.D. thesis, University of Edinburgh) and a protein of larger than 200kD, predicted from the long open reading frame, had been identified in immunoprecipitation experiments. The aim of this project was to raise antisera to those regions of the PRP8 protein not represented in FP8.1 or FP8.2 and to use the new sera, in combination with anti-8.1 and anti-8.2, to analyse the protein. The possession of antisera to all regions of the protein was important for two reasons; (i) the large species may represent a precursor molecule which is cleaved to smaller functional molecule(s) which may not be recognised by antisera raised against FP8.1 or FP8.2, or (ii) even if the large species is the active molecule, it is potentially, due to its size, a multi-domain protein and antisera to all such domains would facilitate functional analysis.

More recently, further sequencing of the 5' end of the PRP8 gene (S.Dunbar, personal communication) has revealed that the start codon lies 351bp upstream from the point predicted by the previous sequence. This means that the protein contains 2413 amino acid residues and has a predicted molecular weight of 280kD.

3.2 RAISING ANTISERA TO PRP8 FUSION PROTEINS

3.2.1 Construction of Plasmids to Synthesise Fusion Proteins

The availability of the full sequence of the PRP8 gene made it possible to determine the regions of predicted coding sequence which were not represented in pFP8.1 and pFP8.2 and, therefore, to construct plasmids to direct synthesis of β-galactosidase fusion proteins containing most of the remainder of the predicted coding sequence. The vectors used for this construction were from the pEX series (Stanley and Luzio, 1984). These contain several unique restriction sites in each of the three reading frames in the 3' end of the lacZ gene which is under the control of the lambda P_r promoter. Therefore, when these plasmids are present in an E. coli strain bearing a lambda cI^{857} prophage which encodes a temperature-sensitive cI repressor protein, synthesis of the β-galactosidase protein, or of the β-galactosidase fusion protein, can be induced by inactivating the cI repressor at 42°C.

Figure 3.1A shows the two regions of the PRP8 gene used to make such constructs; the 1.26kb BamHI-BglII fragment of pY85888 was cloned into the BamHI site of pEX-3 to make pFP8.3 and the 4.15kb BglII fragment of pY85888 was cloned into the BamHI site of pEX-1 to make pFP8.4. The "8.3" fragment consists entirely of PRP8 coding sequence, while the "8.4" fragment consists of 1.46kb of PRP8 coding sequence from the BglII site to the ClaI site, 9bp upstream of the stop codon, and 2.69kb of YCp50 vector sequence. The first in-frame stop codon in the YCp50-derived sequence is 48bp from the ClaI site and, therefore, the plasmid pFP8.4 would be predicted to encode a fusion protein with an extra 16 amino acid residues derived from YCp50 sequence at its C-terminus. The appropriate pEX vectors were selected to maintain the reading frame of the lacZ gene through the PRP8 gene fragment.

E. coli cells carrying plasmids with the correct insert were identified by colony hybridisation, and those with the insert in the desired orientation were identified by screening for fusion protein synthesis. Small-scale protein preparations from such cells were analysed by SDS-PAGE followed by Coomassie Blue staining of the gel. Figure 3.1B shows the results of a screen for FP8.3 synthesis and it is clear that the cell extracts in lanes 1 and 2 contain β-galactosidase (116kD) while that in lane 3 contains a fusion protein with an apparent molecular weight of approximately 175kD. This is

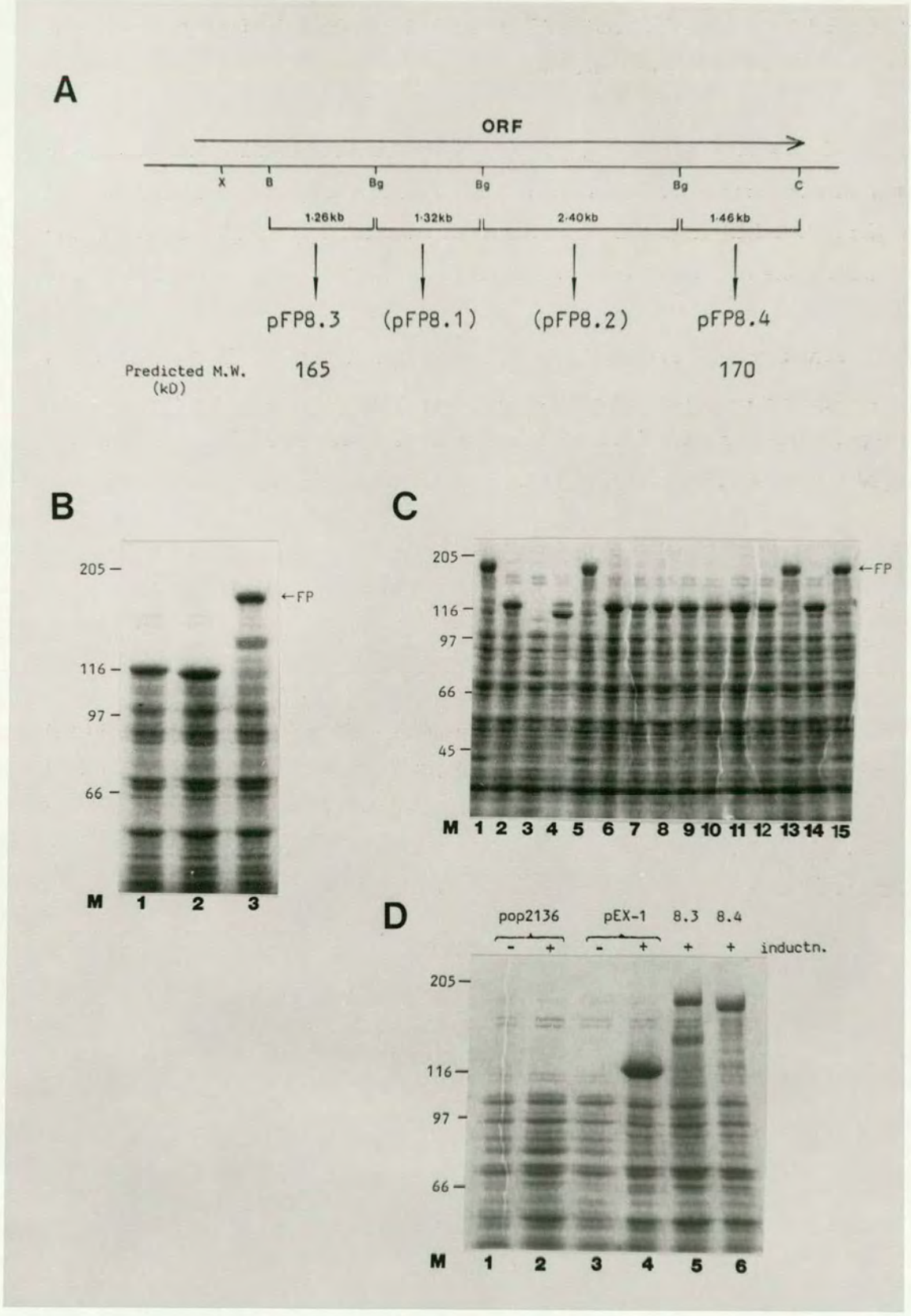
FIGURE 3.1: CONSTRUCTION AND EXPRESSION OF lacZ-PRP8 GENE FUSIONS (8.3 AND 8.4)

(A) Physical map of the PRP8 gene showing the position and orientation of the open reading frame (ORF) and restriction enzyme sites: B, BamHI; Bg, BglII; C, ClaI; X, XbaI. Brackets show the regions used to construct lacZ-PRP8 fusions and the predicted molecular weights of the β-galactosidase-PRP8 fusion proteins are indicated.

(B) Screening for FP8.3 synthesis. Extracts from heat-induced E.coli pop2136 cells containing possible pFP8.3 recombinant plasmids were fractionated on an 8.5% SDS-polyacrylamide gel and stained with Coomassie Blue. Sizes of protein molecular weight markers (M) are indicated (kD).

(C) Screening for FP8.4 synthesis. Extracts from heat-induced E.coli pop2136 cells containing possible pFP8.4 recombinant plasmids were fractionated on an 8.5% SDS-polyacrylamide gel and stained with Coomassie Blue. Markers as in (B).

(D) Coomassie Blue-stained 8.5% SDS-polyacrylamide gel showing extracts from cells of following: pop2136 host strain, uninduced (lane 1) and induced (lane 2); host strain containing pEX-1 vector, uninduced (lane 3) and induced (lane 4); host strain containing pFP8.3, induced (lane 5); host strain containing pFP8.4, induced (lane 6). Markers as in (B).


A
ORF
X
B
Bg
Bg
Bg
C
1·26kb
1·32kb
2·40kb
1·46kb
pFP8.3
(pFP8.1)
(pFP8.2)
pFP8.4
Predicted M.W. (kD)
165
170
B
205
116
97
66
←FP
M 1 2 3
C
205
116
97
66
45
←FP
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
D
pop2136
pEX-1
8.3
8.4
- + - + + + inductn.
205
116
97
66
M 1 2 3 4 5 6

slightly higher than the 165kD predicted by the coding sequence of the PRP8 fragment, but restriction analysis of the plasmid carried in these cells supported the correct structure (data not shown), suggesting that the migration of the fusion protein in SDS-PAGE is aberrantly slow. Figure 3.1C shows a screen for FP8.4 synthesis in which the cell extracts in lanes 2, 6-12, and 14 contain β-galactosidase, while those in lanes 1, 5, 13 and 15 contain a fusion protein with an apparent M.W. of 170kD, which is the size predicted by the coding sequence of the PRP8 fragment. Restriction analysis of the plasmid carried in these cells supported the correct structure (data not shown). Figure 3.1D, by way of summary, shows no change in the protein spectrum in cells of the pop2136 host strain upon incubation at 42°C (compare lanes 1 and 2) while β-galactosidase is synthesised in cells containing the vector pEX-1 upon induction (compare lanes 3 and 4) and the fusion proteins FP8.3 (lane 5) and FP8.4 (lane 6) are synthesised in cells containing the constructs pFP8.3 and pFP8.4, respectively, upon induction.

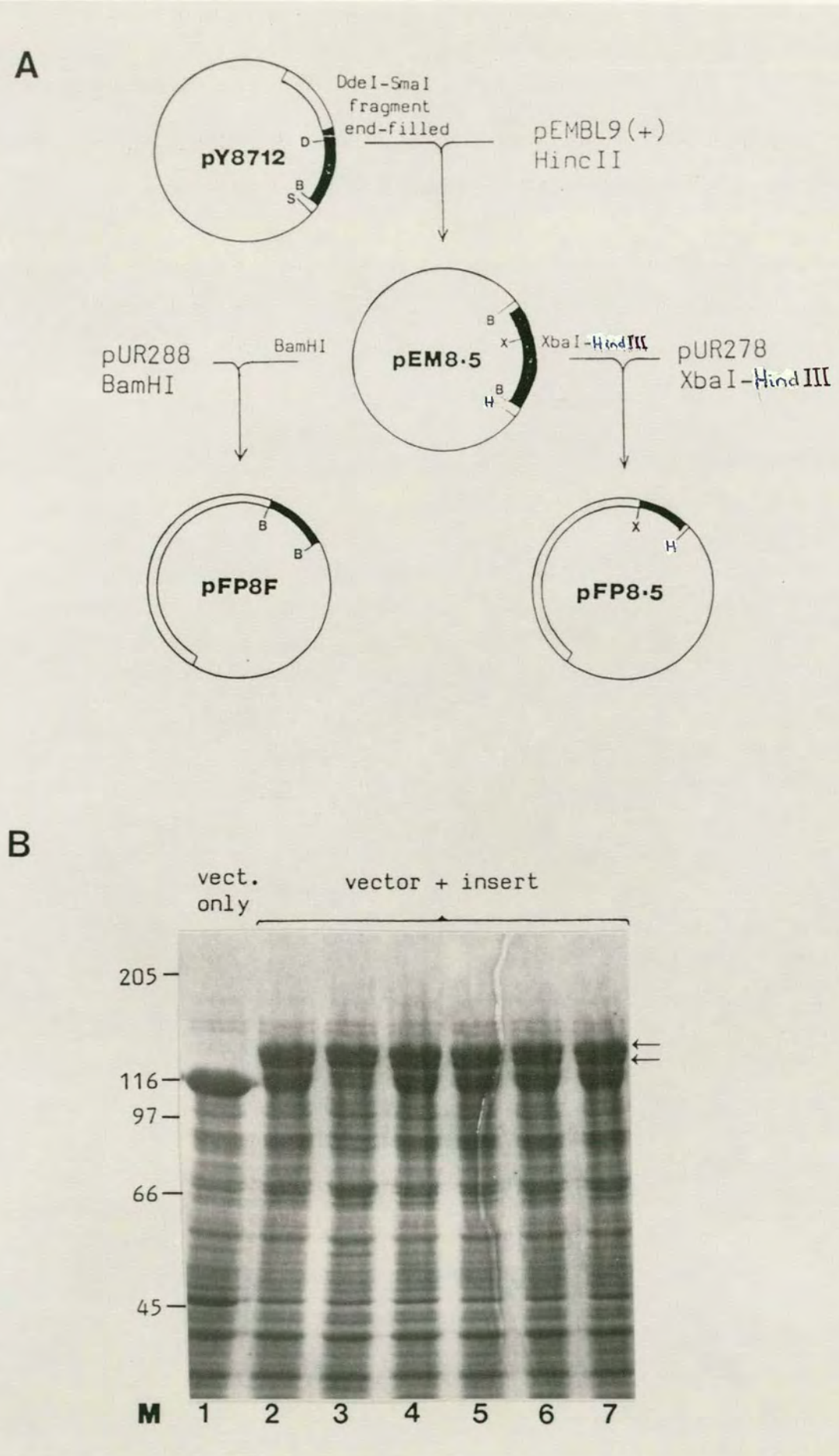
With the discovery that the region at the N-terminus of the PRP8 protein not represented in any of FP8.1-FP8.4 sequences was larger than at first thought (see Section 3.1), it was decided to construct a plasmid which would synthesise a fusion protein containing the rest of the PRP8 protein sequence. In this case, the vector used was from the pUR series (Ruther and Muller-Hill, 1984) which, like the pEX series, have several unique restriction sites in the 3' end of the E. coli lacZ gene, but under the control of its own promoter. These plasmids are propagated in cells carrying the $lacI^q$ allele, on a F' episome, which overproduces the lac repressor protein. Synthesis of the β-galactosidase protein, or the β-galactosidase fusion protein, is induced by growing the cells in the presence of IPTG.

Figure 3.2A depicts the construction of this plasmid. The 0.75kb DdeI-SmaI fragment of pY8712 was subjected to an end-filling reaction and blunt-end ligated into the HincII site in the polylinker of pEMBL9(+). This fragment contains 735bp of PRP8 coding sequence from the DdeI site, 16 codons downstream of the N-terminus of the PRP8 protein, to the BamHI site. Plasmids with the correct insert were identified by restriction analysis of small-scale plasmid preparations, and one of those with the desired orientation of the insert, i.e. with the 5' end of the PRP8 gene fragment oriented toward the EcoRI end of the vector polylinker, was sequenced to

FIGURE 3.2: CONSTRUCTION AND EXPRESSION OF lacZ-PRP8 GENE FUSIONS (8F AND 8.5)

(A) Construction of gene fusions. Restriction enzyme sites: B, BamHI; Bg, BglII; C, ClaI; X, XbaI. The filled box represents PRP8 coding sequence.

(B) Screening for FP8.5 synthesis. Extracts from IPTG-induced E.coli NM522 cells containing pUR278 (lane 1) or pFP8.5 (lanes 2-7) were fractionated in an 8.5% SDS-polyacrylamide gel and stained with Coomassie Blue. Sizes of protein molecular weight markers (M) are indicated (kD).


A
pY8712
D
B
S
DdeI-SmaI
fragment
end-filled
pEMBL9(+)
HincII
pUR288
BamHI
BamHI
pEM8·5
B
X
XbaI-HindIII
H
B
pUR278
XbaI-HindIII
pFP8F
B
B
pFP8·5
X
H
B
vect.
only
vector + insert
205
116
97
66
45
M
1
2
3
4
5
6
7

verify the accuracy of the end-filling and blunt-end ligation reactions (data not shown). This plasmid construct was called pEM8.5.

To make the fusion protein construct, the 0.75kb BamHI fragment of pEM8.5 was cloned into the BamHI site of pUR288 and E.coli cells carrying plasmids with the correct insert were identified by colony hybridisation. However, when 26 of such clones were screened for fusion protein synthesis, they all produced large amounts of β-galactosidase, although several larger proteins, the largest of which had an apparent molecular weight of about 145kD, were faintly detected in some cases (data not shown). This was, in fact, the size predicted by the coding sequence of the PRP8 fragment. When the plasmids in such cells were analysed by restriction digestion, all those which gave faint, large proteins, upon induction, proved to have the correct structure (data not shown). It was concluded that this fusion protein was unstable in E.coli cells and, upon synthesis, the PRP8 portion was rapidly degraded to leave β-galactosidase. This was not altogether surprising, since the N-terminal region of PRP8, which was included in this fusion protein, contains several runs of proline residues, each 6-8 amino acids in length, which would be predicted to confer a very open conformation upon the region and, thus, render it very accessible to proteolytic attack.

As an alternative, a smaller region of the PRP8 gene was used to make a further fusion construct (Fig. 3.2A). The 0.6kb XbaI-HindIII fragment of pEM8.5 was cloned into pUR278, cut with XbaI and HindIII. This fragment contains 546bp of PRP8 coding sequence from the XbaI site, immediately downstream of the last of the runs of proline residues, to the BamHI site. E.coli cells carrying the correct constructs were identified by restriction analysis of small-scale plasmid preparations and screened for fusion protein synthesis. Figure 3.2B shows that all such cells synthesise two major fusion protein species, the larger of which has an apparent molecular weight of approximately 136kD, as predicted from the PRP8 coding sequence. The smaller of the two bands probably represents a species created by the partial degradation of the larger at its C-terminus. This fusion construct was called pFP8.5 and the fusion protein whose synthesis it directs was called FP8.5.

3.2.2 Raising Antisera to the β-Galactosidase Fusion Proteins

Fusion proteins were partially purified from SDS-polyacrylamide gels, or from the insoluble protein fraction, and injected into rabbits. Two rabbits, numbers 56 and 57, were immunised with FP8.3 and two, numbers 58 and 59, with FP8.4. For each immunisation, at least 20μg of the gel-purified fusion protein (denatured) and varying amounts of fusion protein resolubilised from the insoluble fraction (at least partly renatured) were used. Blood withdrawn after the third immunisations of rabbits 56 and 58 yielded sera which detected PRP8 on Western blots (see Section 3.3.2) and so terminal bleeds were collected from these animals after the fourth immunisations. Rabbits 57 and 59 gave significantly poorer responses.

Attempts to raise antisera to FP8.5 in two rabbits failed to produce sera with a significant response against the PRP8 portion of the fusion protein.

3.2.3 Construction of Plasmids to Synthesise TrpE Fusion Proteins

The generation of TrpE fusion proteins, incorporating the same regions of PRP8 as are present in FP8.1-8.5, was undertaken in order to provide large quantities of antigen for affinity purification of PRP8-specific antibodies from the corresponding antisera. The vectors used for these constructions were from the pATH series (Dieckmann and Tzagoloff, 1985), which contain several unique restriction sites, in each of the three reading frames, in the 3' end of the E.coli trpE gene under the control of its own promoter. When these plasmids are present in cells grown in the presence of tryptophan, synthesis of the TrpE protein or TrpE fusion protein is repressed, but lowering the concentration of tryptophan by dilution and the addition of indole acrylic acid leads to induction of synthesis of the protein.

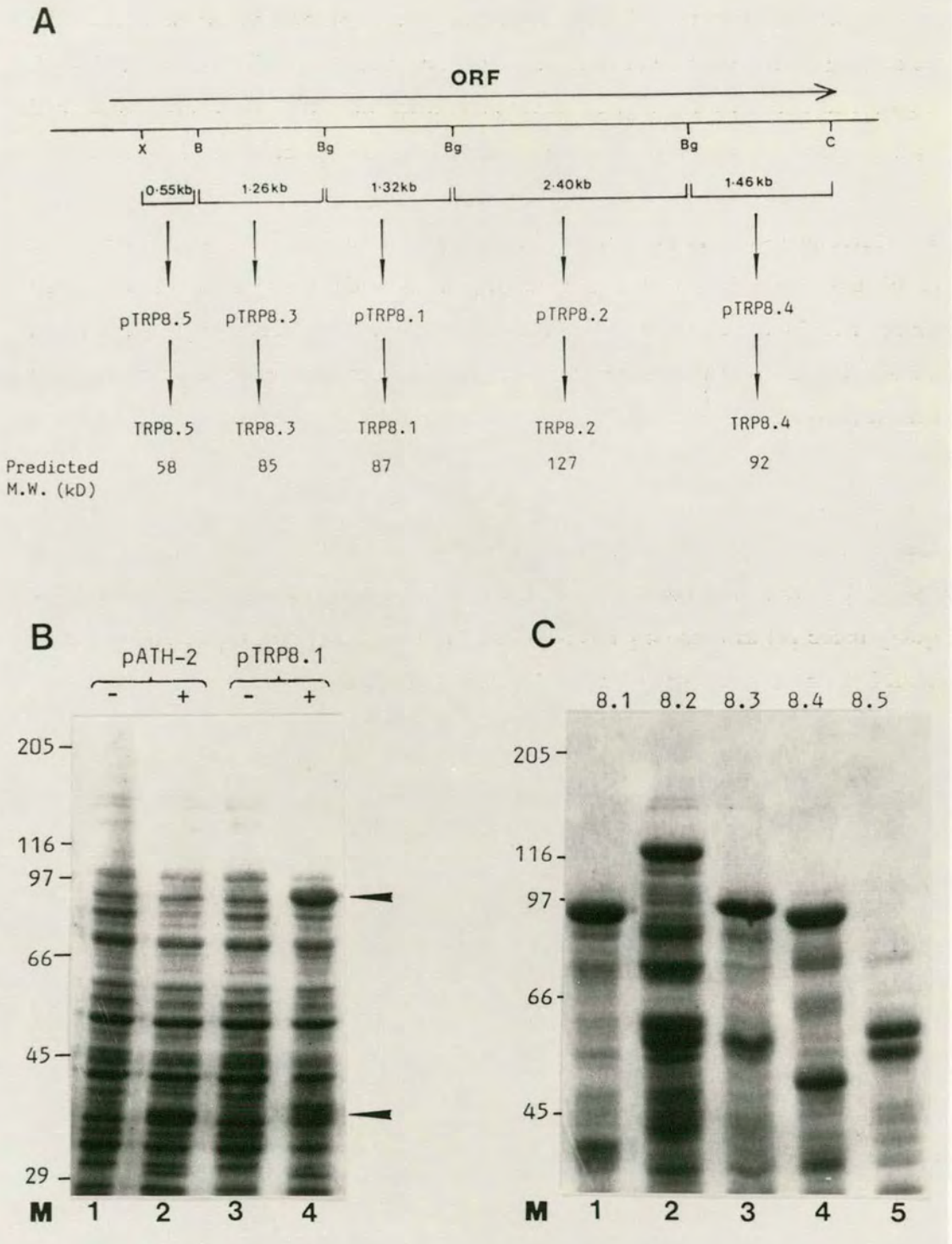
The regions of the PRP8 gene fused to the trpE gene were exactly as for FP8.1-8.5 and are shown in Figure 3.3A. The fusion construct pTRP8.1 consists of the 1.3kb BglII fragment cloned into the BamHI site of pATH-2; pTRP8.2 is the 2.4kb BglII fragment of PRP8 cloned into the BamHI site of pATH-2; pTRP8.3 is the 1.3kb BamHI-HindIII fragment of pFP8.3 (containing the 1.26kb BamHI-BglII fragment of PRP8) cloned between the BamHI and HindIII sites of pATH-2 ; pTRP8.4 is the 1.5kb BglII-ClaI fragment of PRP8 cloned between the BamHI and ClaI sites of pATH-3;

FIGURE 3.3: CONSTRUCTION AND EXPRESSION OF trpE-PRP8 GENE FUSIONS

(A) Physical map of the PRP8 gene showing the position and orientation of the open reading frame (ORF) and restriction enzyme sites: B, BamHI; Bg, BglII; C, ClaI; X, XbaI. Brackets show the regions used to construct the trpE-PRP8 gene fusions and the predicted molecular weights of the TrpE-PRP8 fusion proteins are indicated.

(B) Screening for TRP8.5 synthesis. Extracts from E.coli HB101 cells carrying pATH-2, uninduced (lane 1) or IAA-induced (lane 2), and carrying pTRP8.1, uninduced (lane 3) or IAA-induced (lane 4), were fractionated on a 8.5% SDS-polyacrylamide gel and stained with Coomassie Blue. Sizes of protein molecular weight markers (M) are indicated (kD).

(C) Extraction of TrpE-PRP8 fusion proteins in insoluble protein fractions. Insoluble protein fractions were prepared from IAA- induced E.coli HB101 cells carrying trpE-PRP8 gene fusion constucts and samples were fractionated on an 8.5% SDS-polyacrylamide gel as follows: pTRP8.1 (lane 1), pTRP8.2 (lane 2), pTRP8.3 (lane 3), pTRP8.4 (lane 4), pTRP8.5 (lane 5). Markers as in (B).


A
ORF
X
B
Bg
Bg
Bg
C
0·55kb
1·26kb
1·32kb
2·40kb
1·46kb
pTRP8.5
pTRP8.3
pTRP8.1
pTRP8.2
pTRP8.4
TRP8.5
TRP8.3
TRP8.1
TRP8.2
TRP8.4
Predicted M.W. (kD)
58
85
87
127
92
B
pATH-2
pTRP8.1
- + - +
205
116
97
66
45
29
M 1 2 3 4
C
8.1 8.2 8.3 8.4 8.5
205
116
97
66
45
M 1 2 3 4 5

pTRP8.5 is the 0.6kb XbaI-HindIII fragment of pEM8.6 (containing the 0.55kb XbaI-BamHI fragment of PRP8) cloned between the XbaI and HindIII sites of pATH-2.

E. coli cells carrying plasmids with the correct inserts in the desired orientation were identified by colony hybridisation, by analysis of small-scale protein preparations of induced cultures and by restriction analysis of plasmid DNA preparations (data not shown). Figure 3.3B shows a typical result when total protein from cells containing pATH vector or pTRP8.1, with or without induction, was analysed by SDS-PAGE and Coomassie staining. Upon induction of cells containing vector alone, a protein of approximately 37kD, corresponding to TrpE, was synthesised (compare lanes 1 and 2), while induction of cells containing pTRP8.1 leads to synthesis of a protein of approximately 87kD, as predicted for the TRP8.1 fusion protein (compare lanes 3 and 4).

Figure 3.3C shows a Coomassie-stained SDS-polyacrylamide gel with insoluble protein preparations from E.coli cells induced for synthesis of all five TrpE fusion proteins. TRP8.5 appears as a doublet, with a size difference of approximately 5kD (lane 5), matching the pattern seen with its corresponding β-galactosidase fusion protein. The larger of the two TRP8.5 species and the TRP8.1 (lane 1), TRP8.2 (lane 2) and TRP8.4 (lane 4) proteins all gave apparent molecular weights on SDS-PAGE which were consistent with the sizes predicted from the coding regions of the respective PRP8 fragments. TRP8.3, like the corresponding β-galactosidase fusion protein, migrates aberrantly slowly (lane 3, see Section 3.2.1).

3.3 PROPERTIES OF THE ANTISERA

3.3.1 Immunoprecipitation of PRP8 Protein

A protein larger than 200kD, corresponding to the PRP8 gene product had been immunoprecipitated from L-[^{35}S]-methionine labelled yeast extracts by anti-8.1 serum, but anti-8.2 serum precipitated this protein with a very much lower efficiency (S.Jackson, in Jackson et al., 1988). Figure 3.4 shows such an immunoprecipitation experiment performed with pre-immune and immune 8.3 and 8.4 sera and with immune 8.1 and 8.2 sera. Anti-8.1 serum has precipitated the large PRP8 protein (lane 6) but, in this instance, this protein was not detected in the immunoprecipitation with anti-8.2 serum

FIGURE 3.4: IMMUNOPRECIPITATION OF THE PRP8 PROTEIN

Immunoprecipitation of proteins from L-[^{35}S]-methionine- labelled extract of yeast strain BJ2412 was performed with 10μl of each of the following sera: pre-immune 8.3 (lane 1), immune anti-8.3 (lane 2), pre-immune 8.4 (lane 3), immune anti-8.4 (lane 4), immune anti-8.2 (lane 5), immune anti-8.1 (lane 6). Immunoprecipitates were fractionated in an 8.5% SDS-polyacrylamide gel. Sizes of protein molecular weight markers (M) are indicated (kD).

FIGURE 3.5: DETECTION OF THE PRP8 PROTEIN ON AN IMMUNOBLOT

Extracts were prepared from yeast strain SPJ8.31 containing YEp24 (lanes 1, 3, 5 and 7) or pY8000 (lanes 2, 4, 6 and 8) and samples (approximately 100μg total protein per 0.5cm lane) were electrophoresed through an 8.5% SDS-polyacrylamide gel and electroblotted to nitrocellulose. The blot was probed with antisera as follows; anti-8.3 (lane 1), anti-8.1 (lane 2), anti8.2 (lane 3), anti-8.4 (lane 4). Sizes of protein molecular weight markers (M) are indicated (kD).

FIG 3.4

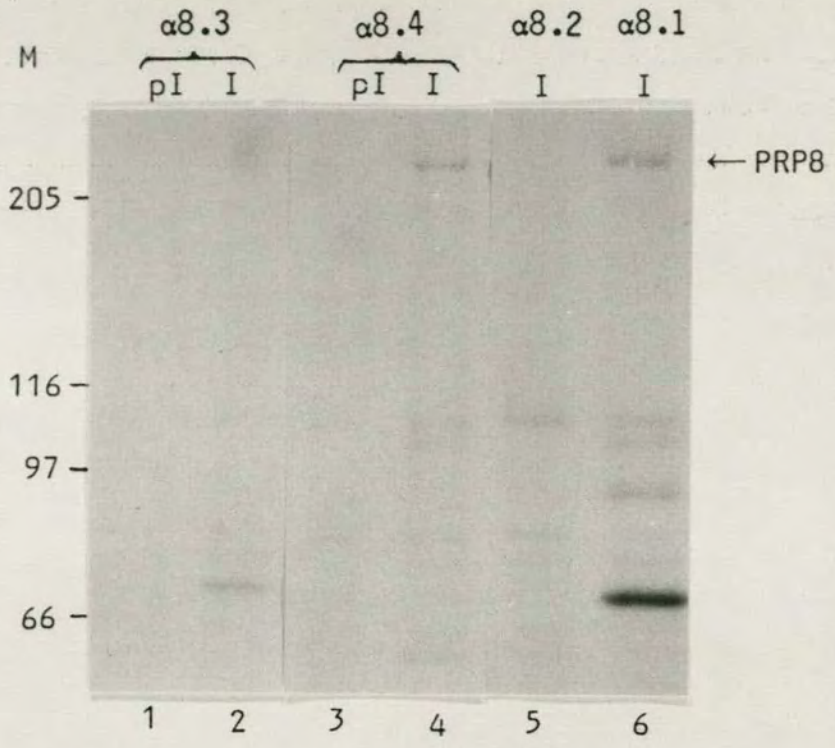
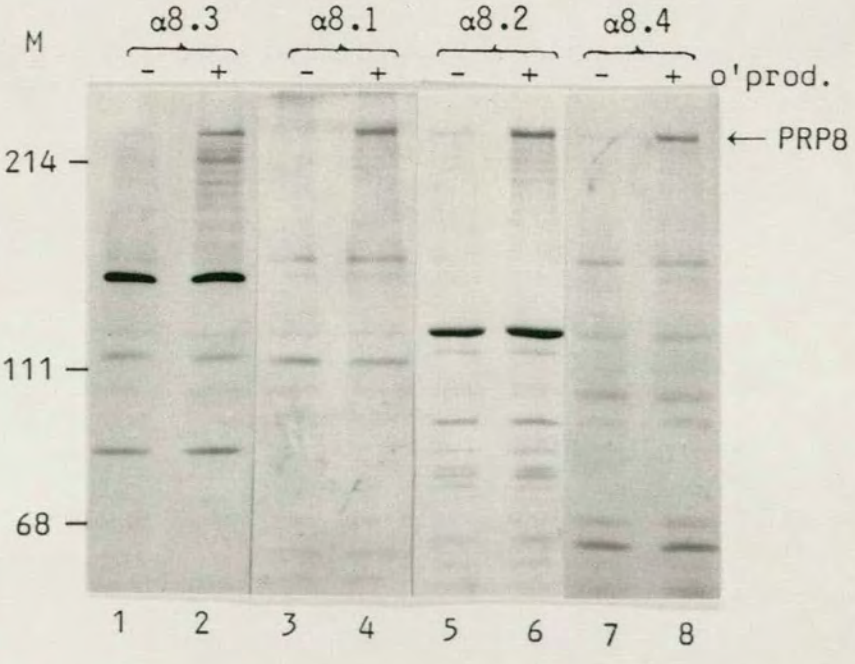

M
α8.3
pI I
α8.4
pI I
α8.2
I
α8.1
I
← PRP8
205
116
97
66
1 2 3 4 5 6

FIG 3.5


M
α8.3
- +
α8.1
- +
α8.2
- +
α8.4
- +
o'prod.
← PRP8
214
111
68
1 2 3 4 5 6 7 8

(lane 5). Pre-immune 8.3 and 8.4 (lanes 1 and 3) and immune 8.3 (lane 2) sera fail to precipitate any labelled proteins of high molecular weight, while immune 8.4 serum immunoprecitates a species which co-migrates with the large PRP8 protein (lane 4). Therefore, both anti-8.1 and anti-8.4 sera are capable of recognising the protein which represents the full-length, primary PRP8 gene product in native protein extracts, whereas anti-8.2 and anti-8.3 cannot do so efficiently. Anti-8.4 serum also immunoprecipitates a protein species of approximately 110kD which co-migrates with that immunoprecipitated by anti-8.1 serum in quantities proportional to the copy number of the cloned PRP8 gene in transformed yeast cells (Jackson et al., 1988). However, this species is not immunoprecipitated with anti-8.4 serum from rabbit no.59, which does immunoprecipitate the full-length PRP8 protein, albeit less efficiently than that from rabbit no.58 (data not shown), and is, therefore, probably not immunologically related to the PRP8 protein.

3.3.2 Detection of the PRP8 Protein by Immunoblotting

Immunoblotting experiments were performed on whole cell extracts from strain SPJ8.31 harbouring the plasmid pY8000 (carrying the PRP8 gene; expected copy number of approximately 5-10 per cell) or the parent vector of pY8000, i.e. YEp24. Figure 3.5 shows the results from an experiment in which such extracts were probed with antisera against the four PRP8 fusion proteins and, clearly, all four detect the 280kD PRP8 protein when it is in the denatured form on a Western blot. The pre-immune sera do not detect this protein (data not shown). The weakness of the signals at 280kD in the extracts from cells carrying YEp24 alone (lanes 1,3,5 and 7) shows that the level of full-length PRP8 protein present in yeast cells is very close to the lower limit of detection by this method (the maximum possible amount of extract was loaded on each lane). The only signal which is enhanced in extracts from cells with the higher copy number of the PRP8 gene is that at 280kD (compare lanes 2, 4, 6 and 8 with lanes 1, 3, 5, and 7 respectively). Therefore, the 110kD protein which is seen in immunoprecipitations (see Section 3.2.3.1) is not detected by any of the antisera on immunoblots, since it, also, was overproduced in cells carrying pY8000.

3.4 DISCUSSION

This chapter has described the generation of antisera against β-galactosidase fusion proteins containing two regions of the PRP8 protein (FP8.3 and FP8.4). These have been characterised by their ability to recognise the 280kD primary product of the PRP8 gene, both by immunoprecipitation and immunoblotting procedures and their properties were compared with those of previously characterised antisera against different PRP8 fusion proteins (FP8.1 and FP8.2).

Anti-8.4 serum was able to precipitate the PRP8 protein, while anti-8.3 serum was not. However, anti-8.3 serum was able to detect the PRP8 protein on immunoblots which suggests that, either the PRP8-specific antibodies in anti-8.3 serum can only recognise denatured epitopes, or that such epitopes are inaccessible to the antibodies in the native protein. This second possibility could be due to the folding of the PRP8 protein itself or to its association with other molecules in complexes. The same phenomenon was seen with anti-8.1 and anti- 8.2 sera, whereby both antisera detect the 280kD PRP8 protein on immunoblots but only anti-8.1 serum immunoprecipitates it efficiently from native extracts (S.Jackson, Ph.D. thesis).

The generation of anti-8.3 and anti-8.4 sera meant that antisera against 90% of the full 280kD PRP8 protein were available to commence a functional analysis of the protein.

CHAPTER 4

THE PRP8 PROTEIN IS A snRNP COMPONENT

4.1 INTRODUCTION

The combination of the anti-PRP8 sera, as described in Chapter 3, and the yeast in vitro splicing system (Lin et al., 1985) provided the means to analyse the function of the PRP8 protein in the splicing reaction. That functional PRP8 protein is required for the pre-mRNA splicing reaction in vitro has been established in two ways: (i) prior immunodepletion of the protein using anti-8.1 and anti-8.2 sera results in a diminution of splicing activity (M.Lossky, in Jackson et al., 1988), (ii) the in vitro splicing activity of extracts from cells carrying the prp8-1 mutation is more heat-labile than that of extracts from wild-type cells (Lustig et al., 1986). This chapter describes the analysis of the interactions of the PRP8 protein with other components of the yeast splicing machinery.

4.2 THE 280kD PRP8 PROTEIN IS ASSOCIATED WITH U5 snRNA

The yeast spliceosome, like its mammalian counterpart, is a large complex containing snRNAs and, presumably, many proteins (see Section 1.7.2). By analogy with the mammalian system, it seems likely that the yeast snRNAs exist outside the spliceosome complexed with proteins as snRNP particles, rather than as naked RNA molecules. To detect any interactions between the PRP8 protein and snRNAs, immunoprecipitation reactions were performed, under low stringency conditions, from in vitro splicing extracts using antibodies from all four anti-PRP8 sera, and any co-precipitated RNA species were purified and 3'-end labelled. By this means, the two forms of U5 snRNA (214 and 179 nucleotides in length) were shown to be co-precipitated when using anti-8.1 or anti-8.4, but not with anti-8.2 or anti-8.3 (M.Lossky, in Lossky et al., 1987). Significantly, U5 snRNA was only precipitated using antibodies from sera which were able to immunoprecipitate the PRP8 protein itself (see Section 3.3.1).

FIGURE 4.1: U5 snRNA CO-PRECIPITATES WITH THE PRP8 PROTEIN

(A) Sonicated extract (lane 1) or splicing extract (lane 2) from yeast strain BJ2412 was subjected to immunoprecipitation with anti-8.1 antibodies, RNA was recovered from the immune complexes, 3'-end labelled and analysed on a 6% polyacrylamide/7M urea gel. DNA size markers (M) were an end labelled MspI digest of pBR322 plasmid DNA (sizes in nucleotides).

(B) Antigen competition. Immunoprecipitation experiments were performed as in (A), but with anti-8.1 antibodies which had been pre-incubated in the presence of an excess of β-galactosidase (lane 1) or of FP8.1 (lane 2).

FIGURE 4.2: THE FULL-LENGTH PRP8 PROTEIN CO-PRECIPITATES WITH snRNAs

Immunoprecipitation from yeast splicing extract was performed using anti-m^3G (lanes 1-4) and anti-8.4 (lane 5) antibodies. Precipitated material was fractionated in a 8.5% SDS-polyacrylamide gel, electroblotted to nitrocellulose and probed with the following antisera; anti-8.3 (lane 1), anti-8.1 (lane 2), anti-8.2 (lane 3), anti-8.4 (lane 4), anti-8.1 (lane 5). Sizes of protein molecular weight markers (M) are indicated (kD). Signals due to antibodies present in the gel are indicated by asterisks.

FIG 4.1

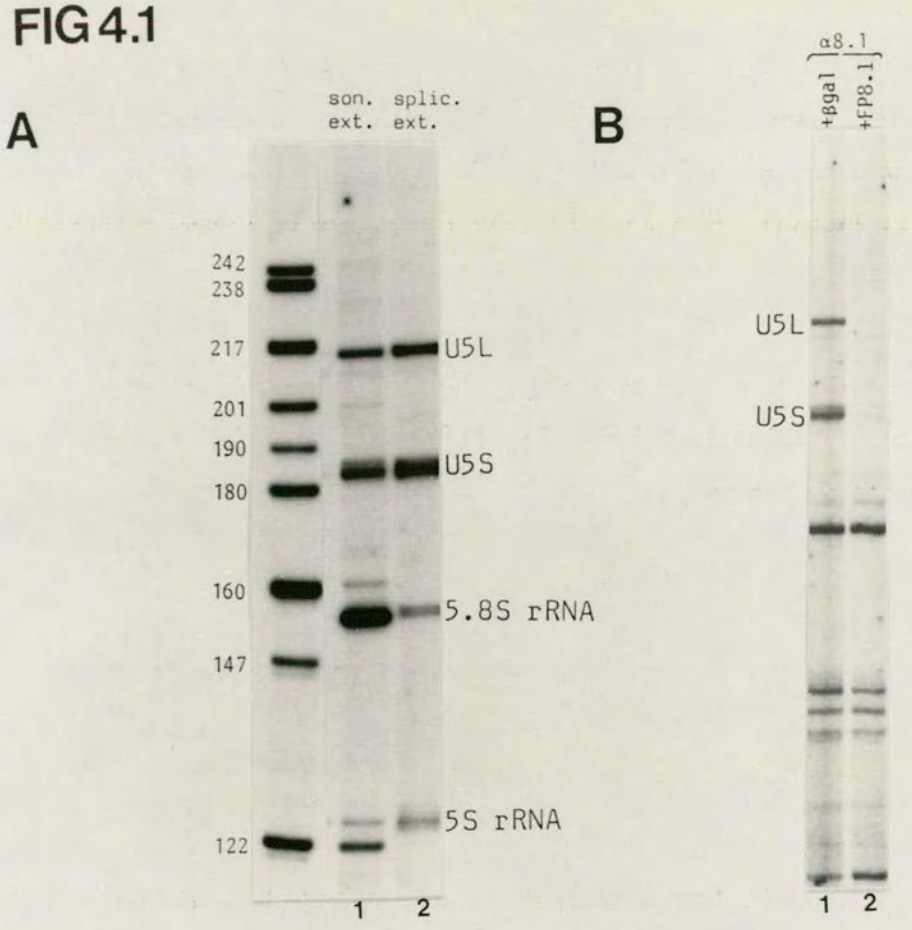
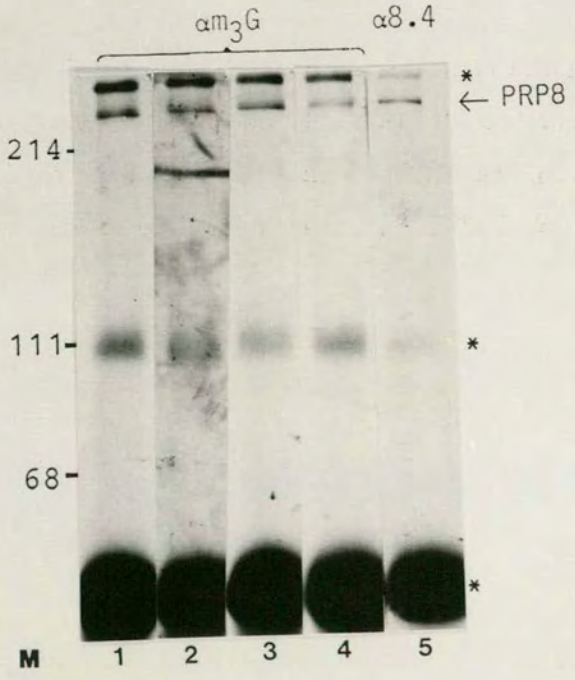

A
son. ext.
splic. ext.
242
238
217
201
190
180
160
147
122
U5L
U5S
5.8S rRNA
5S rRNA
1
2
B
α8.1
+βgal
+fP8.1
U5L
U5S
1
2

FIG 4.2


αm3G
α8.4
* ← PRP8
214
111
68
*
*
M
1
2
3
4
5

To establish that co-precipitation of the U5 snRNA species was due to association with the PRP8 protein and not with other proteins recognised by the antisera, antigen competition experiments were performed. For these, crude, sonicated yeast cell extracts were used, and Figure 4.1A shows that the same two U5 snRNA species were precipitated from this extract (lane 2) as from a splicing extract (lane 1) using anti-8.1 serum. Figure 4.2B shows a competition experiment in which the anti-8.1 antibodies, bound to protein A-Sepharose (PAS), were pre-incubated in the presence of a vast excess of either β-galactosidase (lane 1) or of FP8.1 (lane 2). The difference between these two reactions was that, when competed by β-galactosidase, the PRP8-specific antibodies were still available to bind their epitopes, while competition with FP8.1 blocked these antibodies. It is clear that U5 snRNA was precipitated upon competition with β-galactosidase but not upon competition with FP8.1, while all other labelled RNA species were precipitated to the same extent in both cases, i.e. they represent non-specific background precipitation. This experiment confirmed that the U5 snRNA was precipitated due to an association with the PRP8 protein and similar results were subsequently obtained when antigen competition experiments were performed on splicing extracts (see Section 4.3.1).

Antibodies against the m^3G cap structure can be used to precipitate the full range of snRNAs from yeast cell extracts, including the two forms of U5 (Riedel et al., 1986). Therefore, the proteins which were co-precipitated with snRNAs from a splicing extract were analysed by SDS-PAGE and Western blotting. The results are shown in Figure 4.2, where lane 5 shows detection of the 280kD PRP8 protein when probed with anti-8.1 serum following precipitation with anti-8.4 antibodies and lanes 1-4 show material precipitated with anti-m^3G antibodies and probed with each of the four anti-PRP8 sera. The asterisks indicate signals which are due to the antibodies in the gel, i.e. these were detected in controls where the IgG-PAS complexes were denatured and run on the SDS-gel without incubation with splicing extract (data not shown). The only specific signal detected in the anti-m^3G precipitate corresponds to a protein of 280kD detected by all four anti-PRP8 sera and which co-migrates with that precipitated by anti-8.4 antibodies. It appears, then, that it is only the full-length, 280kD PRP8 protein which is associated with the U5 snRNA and not a smaller processed product, since no other PRP8-specific band is detected on the Western blots.

4.3 EFFECTS OF ATP ON snRNP PRECIPITATION

4.3.1 Incubation with ATP Has Two Effects on snRNP Precipitation

M. Lossky (in Lossky et al., 1987) was able to show two distinct effects on the pattern of snRNAs associated with the PRP8 protein upon incubation of splicing extract under in vitro splicing conditions, without added substrate pre-mRNA ("mock-splicing" conditions), in the presence of ATP compared to when ATP was absent. Figure 4.3 shows such an experiment with anti-8.4 antibodies. Firstly, there was an increase in the amount of the two forms of U5 snRNA which was precipitated and, secondly, there was additional precipitation of U4 snRNA when ATP was present (compare lanes 1 and 2). The additional precipitation of U4 snRNA was shown to be specific to anti-PRP8 antibodies by a competition experiment in which it is abolished by pre-saturation of the antibodies with FP8.4 (lane 4), but not by pre-saturation with β-galactosidase (lane 3). With anti-8.1 antibodies, the increased precipitation of U5 snRNA is observed, but not the additional precipitation of U4 snRNA (M.Lossky, in Lossky et al., 1987).

4.3.2 Increased U5 snRNP Precipitation

The increased precipitability of U5 snRNA upon incubation under mock-splicing conditions in the presence of ATP could be due to either; (i) de novo association of PRP8 protein with U5 snRNAs, or (ii) increased precipitability of PRP8-U5 snRNP complexes which already exist in the extract. To test this, the efficiency of precipitation of the PRP8 protein with anti-8.4 antibodies was compared before and after incubation of a splicing extract in the presence of ATP. Figure 4.4A shows that precipitation of the PRP8 protein, detected by Western blotting, was increased approximately 3-fold after incubation with ATP, reflecting the increased precipitation of the U5 snRNA species after incubation of the same extract with ATP (Figure 4.4B). In similar experiments, the amount of PRP8 protein precipitated from an extract which had been incubated under mock-splicing conditions in the absence of ATP was increased relative to that precipitated from extract which had not been pre-incubated, but was less than that precipitated from extract incubated in the presence of ATP (data not shown). This, once again paralleled the increased precipitability of the U5 snRNA species and,

FIGURE 4.3: EFFECT OF INCUBATION WITH ATP ON snRNA PRECIPITATION

Splicing extract was incubated at 25°C for 20 minutes in splicing buffer without ATP (lane 1) or in the presence of 2mM ATP (lanes 2-4), before being subjected to immunoprecipitation with anti-8.4 antibodies and RNA was purified and 3'-end labelled. Antigen competition was performed by pre-treatment of the antibodies with an excess of β-galactosidase (lane 3) or FP8.4 (lane 4).

FIGURE 4.4: INCUBATION WITH ATP INCREASES THE PRECIPITABILITY OF PRP8

(A) Splicing extract was subjected to immunoprecipitation with anti-8.4 antibodies directly (lanes 2, 4, 6 and 8) or after incubation in splicing buffer with 2mM ATP (lanes 3, 5, 7 and 9). Precipitated material was fractionated in an 8.5% SDS-polyacrylamide gel and blotted to nitrocellulose. Mock immunoprecipitation was performed in the absence of extract (lane 1). The blot was probed with antisera as follows; anti-8.3 (lanes 1-3), anti-8.1 (lanes 4 and 5), anti-8.2 (lanes 6 and 7) and anti-8.4 (lanes 8 and 9). Sizes of protein molecular weight markers (M) are indicated (kD).

(B) Splicing extract was subjected to immunoprecipitation with anti-8.4 antibodies directly (lane 1) or after incubation at 25°C for 20 minutes in splicing buffer with 2mM ATP (lane 2) and precipitated RNA was purified and analysed as described in Figure 4.1.

FIG 4.3

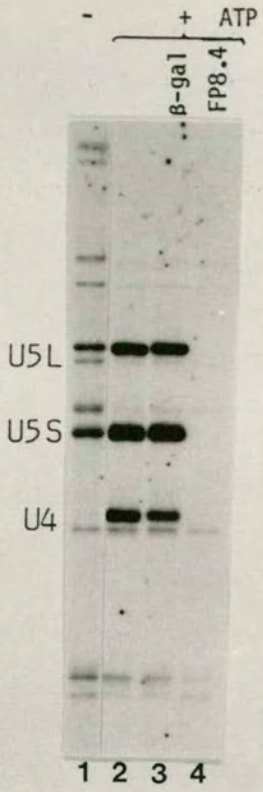
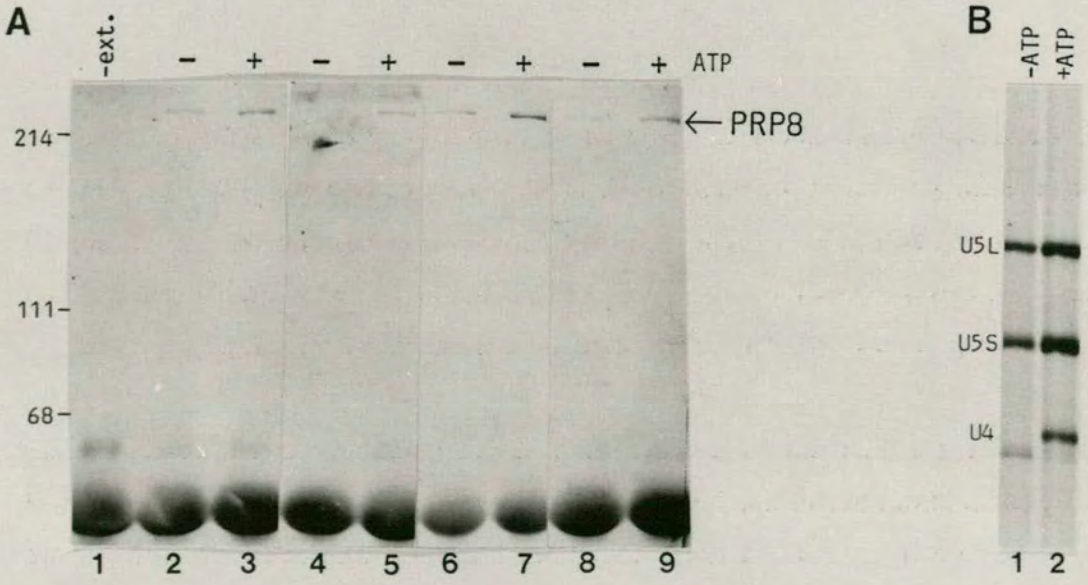

-
+ ATP
β-gal
FP8.4
U5L
U5S
U4
1 2 3 4

FIG 4.4


A
-ext.
- + - + - + - + ATP
214
111
68
← PRP8
1 2 3 4 5 6 7 8 9
B
-ATP
+ATP
U5L
U5S
U4
1 2

presumably, reflected the presence of endogenous ATP in the splicing extract. Indeed, the increase in U5 snRNA precipitability upon incubation without additional ATP varies greatly between different extracts, suggesting that the amount of endogenous ATP present is highly variable.

These results indicate, then, that the increased precipitability of the U5 snRNA species, upon incubation in the presence of ATP, reflects an increased precipitability of U5 snRNP particles which contain the 280kD PRP8 protein.

4.3.3 Precipitation of U4 and U6 snRNAs

It was shown that incubation of splicing extract under mock-splicing conditions in the presence of ATP leads to precipitation of U4 snRNA with anti-8.4 antibodies (Section 4.3.1) and that this represented U4 and U5 snRNPs precipitating as a complex rather than as separate snRNPs independently associated with the PRP8 protein (M.Lossky, in Lossky et al., 1987). In metazoan cells, the U4 and U6 snRNAs are found base-paired to one another in a single snRNP particle (Bringmann et al., 1984; Hashimoto and Steitz, 1984; Rinke et al., 1985) while the yeast species have also been shown to be associated by base-pairing (Siliciano et al., 1987; Brow and Guthrie, 1988). This suggested that U6 snRNA may also be associated with PRP8 after incubation in the presence of ATP, yet no species of the appropriate size was detected in 3'-end labelling experiments. However, it was possible that this was due to the U6 snRNA being a poor substrate for the 3'-end labelling reaction, since its 3' terminus contains a run of uridine residues and is predicted to be sequestered in secondary structure (England et al., 1980).

Possible precipitation of U6 snRNA was investigated by Northern blot analysis. RNA, precipitated from a splicing extract with antibodies from anti-8.4 serum, was purified. Half was 3'-end labelled and analysed as usual, while the other half was electrophoresed, unlabelled, through a denaturing polyacrylamide gel, electroblotted to a nylon membrane and probed with a radiolabelled oligonucleotide complementary to U6 snRNA. Figure 4.5 depicts the result of such an experiment in which panel A shows the Northern blot probed for U6 and panel B shows the 3'-end labelled RNA from the same reactions. Clearly, U6 snRNA (112-115nt) was precipitated by anti-8.4 antibodies after incubation in the presence of ATP (compare lanes 2 and 3). The

FIGURE 4.5: U6 snRNA CO-PRECIPITATES WITH THE PRP8 PROTEIN

(A) Splicing extract was incubated at 25°C for 20 minutes in splicing buffer without ATP (lane 2) or in the presence of 2mM ATP (lanes 3-5) before being subjected to immunoprecipitation with anti-8.4 antibodies and RNA was purified from the immune complexes. The samples were split and half of each was fractionated in a 6% polyacrylamide/7M urea gel. Total RNA (T) extracted from from a half volume of splicing extract was directly analysed (lane 1). RNA was electroblotted to Hybond N nylon membrane and probed with a 5'-end labelled U6 snRNA- specific oligonucleotide. Antigen competition was performed by pre-incubation of the antibodies with an excess of β-galactosidase (lane 4) or FP8.4 (lane 5).

(B) The other half of the purified RNA from each of the reactions described in (A) was 3'-end labelled and analysed on a 6% polyacrylamide/7M urea gel.

FIGURE 4.6: EFFECT OF HEPARIN ON snRNA PRECIPITATION

Splicing extract was incubated at 25°C for 20 minutes in splicing buffer without ATP (lanes 1 and 3) or in the presence of 2mM ATP (lanes 2 and 4). Distilled H_2O (1/10th volume; lanes 1 and 2) or heparin (1/10th volume of 40mg/ml; lanes 3 and 4) was added and incubation continued for a further 5 minutes. The reaction mixes were subjected to immunoprecipitation with anti-8.4 antibodies and the precipitated RNA was analysed as described in Figure 4.1.

FIG 4.5

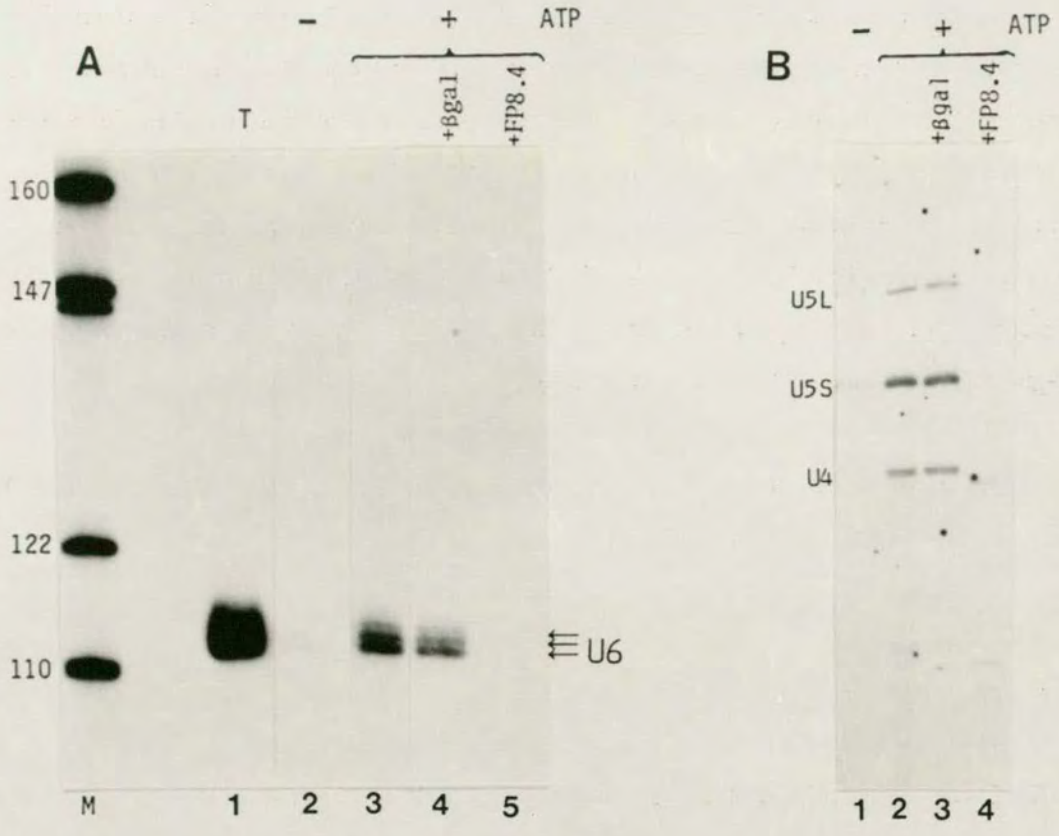
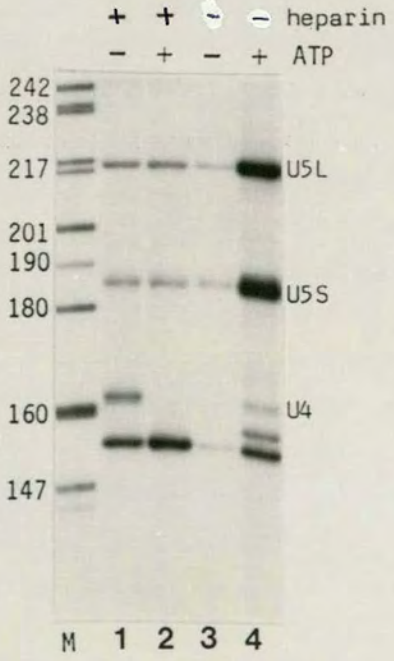

A
B
ATP
T
+βgal
+FP8.4
160
147
122
110
U6
U5L
U5S
U4
M
1
2
3
4
5

FIG 4.6


heparin
ATP
242
238
217
201
190
180
160
147
U5L
U5S
U4
M
1
2
3
4

abolition of U6 snRNA precipitation by pre-incubation of the antibodies with an excess of FP8.4 (lane 5), but not by pre-incubation with an excess of β-galactosidase (lane 4), shows that the precipitation of U6 snRNA is mediated by the PRP8 protein. Antibodies from anti-8.1 serum do not precipitate U6 snRNA (data not shown), and so the pattern of precipitation of this species exactly parallels that of the U4 snRNA.

4.4 EFFECTS OF HEPARIN ON snRNP PRECIPITATION

The association of U4/U6 and U5 snRNPs upon incubation in the presence of ATP (as described in Section 4.3) appeared to be in direct contradiction to the results of Cheng and Abelson (1987), who report an ATP-dependent dissociation of an endogenous U4/U5/U6 snRNP complex, as analysed by electrophoresis on non-denaturing polyacrylamide gels. However, it was possible that the addition of heparin to the samples, prior to loading on the gel, was having an effect on the complexes. To test this, immunoprecipitations with anti-8.4 antibodies were performed in the presence of an equivalent amount of heparin (final concentration of 4mg/ml). The result is shown in Figure 4.6, and, although some of the sample has been lost from lane 3 (compare background signals with those in other lanes), it is clear that the presence of heparin has dramatic effects on the precipitation of snRNAs. Firstly, in the presence of ATP, the amount of U5 snRNA precipitated is greatly decreased (compare lanes 2 and 4) and, secondly, U4 snRNA is precipitated in the absence of ATP (lane 1), but not in its presence (lane 2). It appears that the presence of heparin disrupts the U5 snRNP, such that it is only poorly precipitated with anti-8.4 antibodies, and causes it to associate with U4 snRNA (presumably as part of the U4/U6 snRNP) in a complex which is dissociated in the presence of ATP. These results, in the presence of heparin, are entirely consistent with those obtained with the non-denaturing gel system (Cheng and Abelson, 1987).

4.5 IS PRP8 AN Sm-ANTIGEN?

The finding that PRP8 is a stable component of the yeast U5 snRNP raises the possibility that the precipitability of the U5 snRNP by antibodies from various Sm-sera (see Section 1.5.2.2) occurs via a direct recognition of this particular protein. To test

this, Western blots of total yeast splicing extract were probed with various Sm-sera (Kung, JE and #24) but, while they all detected a variety of smaller species, none detected a protein of greater than 200kD (data not shown). In addition, when blots of material precipitated from splicing extracts by anti-8.4 antibodies were probed, no signals were detected (data not shown). These results seem to indicate that PRP8 is not an Sm-antigen, but could also mean that the Sm-antibodies do not recognise the epitopes in a denatured form or, indeed, that insufficient PRP8 is present on the blots to be detected.

4.6 DISCUSSION

In this chapter, it has been demonstrated that the full-length, 280kD, PRP8 protein is a component of the U5 snRNP. Under a variety of conditions which affect the precipitability of U5 snRNA species, the long (214nt) and short (179nt) forms are always precipitated in approximately equal amounts (M.Lossky, personal communication). This suggests that the two forms exist within a single snRNP particle, or that there are two forms of the U5 snRNP which are present in approximately equal amounts, both containing the PRP8 protein (for further discussion, see below). Variations in the precipitability of the PRP8 protein appear to be reflected in the precipitability of the U5 snRNA species, indicating that the PRP8 protein is a stable component of the snRNP particle. This is further supported by the finding that the association is observed in crude, sonicated cell extracts which are not competent for the <u>in vitro</u> splicing reaction.

The failure of anti-8.2 and anti-8.3 antibodies to precipitate the U5 snRNA species reflects their inability to precipitate the PRP8 protein from [^{35}S]-labelled cell extracts (see Section 3.3.1). As was suggested in Section 3.4, this could be due to an inability to recognise their epitopes in the native protein or to the inaccessibility of their epitopes. Neither possibility can be discounted, although the finding that the PRP8 protein is a stable snRNP component, complexed with snRNA and, presumably, other proteins, might suggest that certain regions of the protein would be masked by these other components and rendered inaccessible to antibody binding.

The increased precipitability of the U5 snRNP following incubation of splicing extract in the presence of ATP can be explained in either of two ways. Firstly, it may

be due to a conformational change in the PRP8 protein, or in the snRNP particle, such that the anti-8.1 and anti-8.4 epitopes are rendered more accessible to antibody binding. Alternatively, the ATP incubation may result in the release of the U5 snRNP from higher order complexes in which these epitopes are inaccessible to antibody binding. Given that the increased precipitability is observed with both anti-8.1 and anti-8.4 antibodies, which recognise large and widely separated (in the primary sequence) regions of the PRP8 protein, any conformational change would need to be very substantial and, therefore, the second explanation would seem more plausible.

The additional co-precipitation of U4 and U6 snRNAs, by anti-8.4 antibodies, upon incubation in the presence of ATP, would appear to represent the association of U5 and U4/U6 snRNPs to form a multi-snRNP complex. Anti-8.1 antibodies do not precipitate U4 and U6 snRNAs from extracts incubated with ATP, even though precipitation of U5 snRNA is very efficient. Immunodepletion of U5 snRNPs from an ATP-treated extract using anti-8.1 antibodies prevents the subsequent precipitation of both U5 and U4 snRNAs by anti-8.4 antibodies (M.Lossky, personal communication). This result suggests that the binding of anti-8.1 antibodies disrupts the U4/U5/U6 snRNP complex and precipitates all the released U5 snRNP and that anti-8.4 antibodies do not recognise the U4/U6 snRNP directly.

Recently, antibodies against the yeast PRP4 protein have been shown to precipitate complexes containing U4, U5, and U6 snRNAs from splicing extracts (Peterson-Bjorn et al., 1989; Banroques and Abelson, 1989). Such complexes are precipitated in the absence of exogenous ATP, although at higher salt concentrations U4 and U6 are preferentially precipitated and in the presence of heparin and ATP only U4 and U6 snRNAs are precipitated (Banroques and Abelson, 1989), suggesting that the PRP4 protein is associated with the U4/U6 snRNP. It may be that endogenous complexes containing U4, U5 and U6 snRNPs are precipitable by antibodies against the PRP4 protein, but not by those against the PRP8 protein. These results, and those described in this chapter, can be drawn together into a model wherein endogenous snRNP complexes dissociate upon incubation in the presence of ATP, freeing the U4/U6 and U5 snRNPs to interact with each other in a distinct complex, precipitable by anti-8.4 antibodies. The results with anti-PRP4 antibodies in the presence of heparin are similar to those obtained with anti-PRP8 antibodies, i.e. complexes

containing U4, U5, and U6 snRNA are present in the absence of ATP, but are disrupted in its presence.

Studies have been carried out on the formation of snRNP complexes, in the absence of heparin, in HeLa cell extracts. Konarska and Sharp (1987, 1988) detected an endogenous U4/U5/U6 multi-snRNP particle in HeLa cell nuclear extracts, as assayed by nondenaturing gel electrophoresis. However, upon incubation in the presence of ATP, more free U4, U6 and U4/U6 snRNP particles were detected and U4/U5/U6 multi-snRNP particles accumulated. The endogenous complex may not be functionally equivalent to the U4/U5/U6 multi-snRNP particle. Possibly, upon incubation with ATP, snRNPs are released from such an endogenous complex and become free to form into U4/U5/U6 multi-snRNP complexes. Such a model would be consistent with the results described in this chapter for yeast extracts. D. Black (personal communication) analysed snRNP complex formation in HeLa cell extracts by means of glycerol gradient centrifugation and his data clearly indicate the ATP-dependent formation of a U4/U5/U6 snRNP complex. Endogenous multi-snRNP complexes were not detected by this method.

The ATP-dependent U4/U5/U6 snRNP complex, detected in immunoprecipitation reactions, may represent an intermediate in spliceosome formation, a pathway for which has been deduced from the analysis of snRNA components of complexes separated on non-denaturing gels (Pikielny et al., 1986; Konarska and Sharp, 1987). The first splicing-specific complex which forms on the pre-mRNA contains U2 snRNA and this is converted to one which contains U2, U4, U5, and U6 snRNAs. Therefore, the formation of the U4/U5/U6 multi-snRNP complex is entirely compatible with these snRNPs entering the spliceosome simultaneously. That the level of the U4/U5/U6 snRNP complex in HeLa cell extracts declines as active spliceosomes are formed (Konarska and Sharp, 1987), lends support to the idea that this complex represents an intermediate in spliceosome assembly.

Since the PRP8 protein is a stable component of the U4/U5/U6 multi-snRNP complex, it seems likely that this protein, also, is incorporated into the spliceosome. Indeed, it was shown that anti-8.4 antibodies, but none of those against other fusion proteins, were able to precipitate splicing intermediates (free exon 1 and lariat intron-exon 2) and one of the products (lariat intron) from a yeast in vitro splicing reaction (M.Lossky, personal communication). This indicates that the PRP8 protein is,

indeed, present in the functional spliceosome and that, following partial dissociation of the complex after the reaction, it remains associated with the lariat intron product and not with the spliced mRNA.

As mentioned above, both forms of the yeast U5 snRNA were always precipitated in approximately equal amounts using anti-PRP8 antibodies. Analysis of the snRNA components of the yeast spliceosome yielded similar results, i.e. both forms were detected in approximately equal amounts (Pikielny et al., 1987). Taken together with the results presented in this chapter, several possible models for the existence of two forms of this species can be postulated; (i) each U5 snRNP contains equal amounts of each form, (ii) each U5 snRNP contains either one form or another, but each multi-snRNP complex or spliceosome contains equal amounts of each form of U5 snRNP, or (iii) as for (ii), but each multi-snRNP complex spliceosome contains either a U5 snRNP with the long form of snRNA or one with the short form, i.e. the two forms are functionally interchangeable. Patterson and Guthrie (personal communication) have shown that yeast cells carrying a mutated SNR7 gene, capable only of transcribing the short form of U5 snRNA, are viable. This result supports the idea that the two forms are functionally interchangeable, although it cannot be discounted that such cells would be disadvantaged under certain growth conditions.

CHAPTER 5

INTERACTIONS OF THE PRP8 PROTEIN

5.1 INTRODUCTION

In Chapter 4, it was established that the PRP8 protein is a stable component of the U5 snRNP and that it enters the spliceosome, possibly via a U4/U5/U6 multi-snRNP complex. With the antibodies against β-galactosidase-PRP8 fusion proteins, several routes to the investigation of interactions of the PRP8 protein within these complexes are available. In this chapter, attempts to reconstitute the U5 snRNP are described, the success of which would allow identification of the protein components of the particle and analysis of RNA-protein interactions. In addition, the RNA-binding properties of the PRP8 protein, and of fusion proteins containing regions of the PRP8 protein, are investigated.

5.2 IN VITRO RECONSTITUTION OF THE U5 snRNP

5.2.1 Synthesis of SNR7 Gene

In vitro assembly of functional U1 snRNPs on in vitro transcribed, radiolabelled U1 snRNA has been achieved in Xenopus oocyte extract (Hamm et al., 1987) and in HeLa cell S100 extract (Patton et al., 1987). This led to a detailed analysis of the RNA-protein interactions in this complex, and to structural models for the snRNP (Hamm et al., 1988; Patton and Pederson, 1988).

To perform analogous experiments for the yeast U5 snRNP, it was necessary to generate yeast U5 snRNA transcripts in vitro. However, the cloned SNR7 gene (Patterson and Guthrie, 1987) has no convenient restriction sites at its 5' or 3' ends to allow its insertion downstream of a bacteriophage promoter (SP6 or T7). To circumvent this problem, it was decided to synthesise the gene de novo. Six oligonucleotides, as shown in Figure 5.1A, were synthesised and the full-length products were gel-purified. These were designed to produce, when ligated together, the SNR7 gene with the

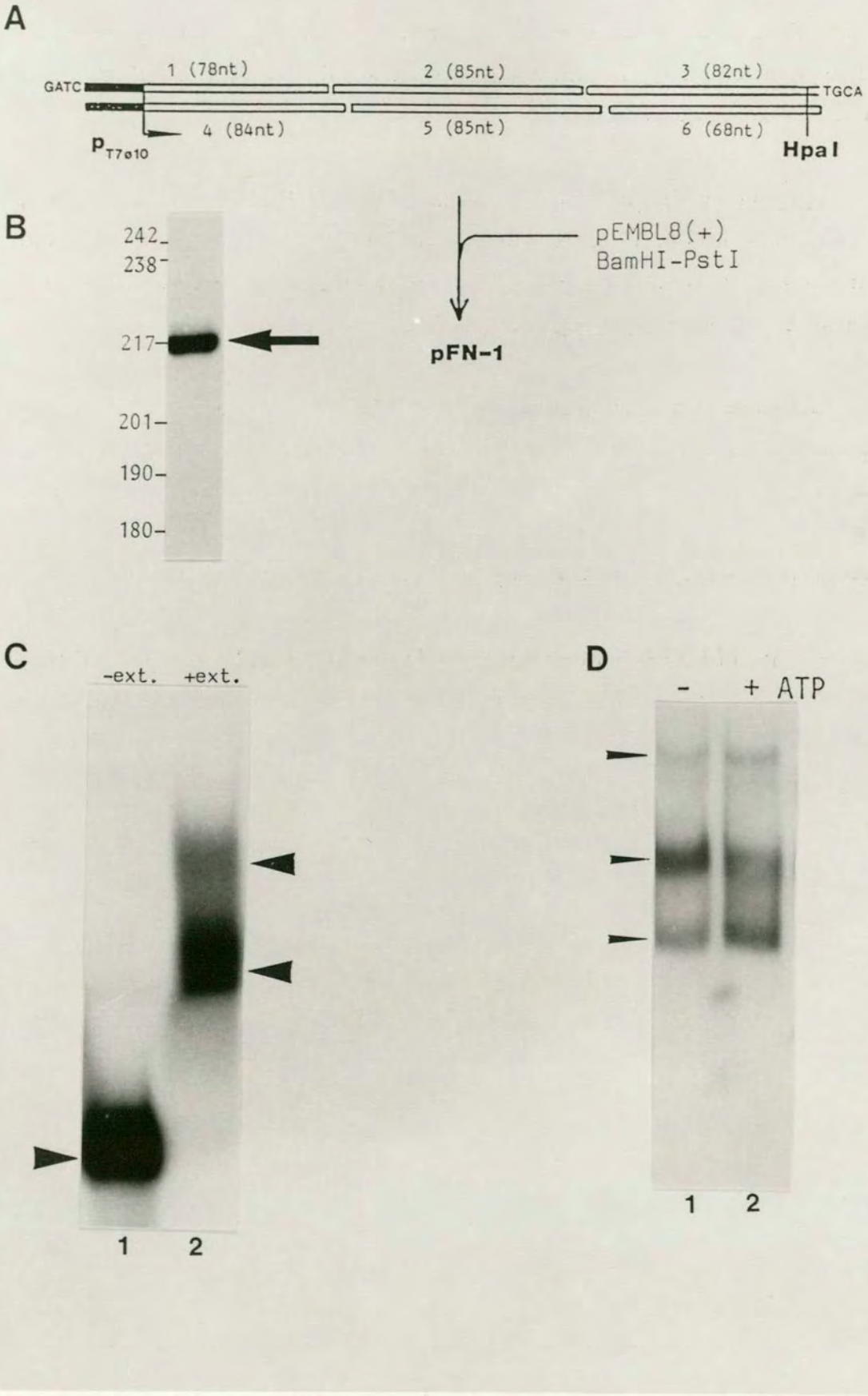
FIGURE 5.1: ATTEMPTED IN VITRO RECONSTITUTION OF THE U5 snRNP

(A) Construction of the synthetic SNR7 gene. The six oligonucleotides used to construct the gene are indicated, showing their sizes and the positions of the phage T7 gene 10 promoter, the HpaI restriction enzyme site and the BamHI and PstI restriction enzyme site compatible termini.

(B) In vitro transcription of the SNR7 gene. The plasmid pFN-1 was linearised with HpaI and transcribed by T7 RNA polymerase and an aliquot of the reaction mix was electrophoresed through a 6% polyacrylamide/7M urea gel. Sizes of markers are indicated in nucleotides (MspI digest of pBR322 plasmid DNA).

(C) Gel retardation assay for protein binding to U5 snRNA. Radiolabelled, in vitro transcribed U5 snRNA was incubated for 15 minutes at room temperature in the presence of 75mM monovalent cations, 5mM $MgCl_2$, 2mg/ml tRNA in the absence (lane 1) or presence (lane 2) of 5μl of yeast splicing extract. The reaction mixes were electrophoresed through non-denaturing composite polyacrylamide/agarose gels.

(D) Analysis of U5 snRNA-containing complexes in a yeast splicing extract. Extract was incubated at 25°C for 20 minutes in splicing buffer in the absence of ATP (lane 1) or in the presence of 2mM ATP (lane 2). The reaction mixes were electrophoresed through a non-denaturing gel, as in (C), blotted to Hybond N nylon membrane and probed with a 5'-end labelled oligonucleotide specific for U5 snRNA.


A
1 (78nt)
2 (85nt)
3 (82nt)
GATC
TGCA
4 (84nt)
5 (85nt)
6 (68nt)
$p_{T7\phi10}$
HpaI
B
242
238
217
201
190
180
pEMBL8(+)
BamHI-PstI
pFN-1
C
-ext.
+ext.
1
2
D
-
+ ATP
1
2

bacteriophage T7 gene 10 promoter at its 5' end and a HpaI restriction site at its 3' end in a BglII-PstI cassette. This was cloned into the BamHI and PstI sites of pEMBL8(+) and single-stranded DNA was prepared and sequenced. This verified that the construct had the desired sequence, and the plasmid was named pFN-1.

When pFN-1 plasmid DNA was linearised by digestion with HpaI, it could be used as a template for in vitro transcription using T7 RNA polymerase. The 217nt run-off transcript synthesised in this way was identical to the long-form of U5 snRNA except that it possessed 2 additional G residues at the 5' end and an additional U residue at the 3' end. Figure 5.1B shows the [^{32}P]-labelled transcript obtained in such a reaction, where essentially all of the RNA synthesised is full-length.

5.2.2 Attempted Reconstitution of U5 snRNP

Radiolabelled, in vitro synthesised U5 snRNA was incubated in the presence of cold, competitor tRNA and yeast cell extract and binding of proteins was analysed by gel retardation assay on non-denaturing polyacrylamide-agarose composite gels. Figure 5.1C shows the result of a typical experiment where lane 1 contained RNA in the absence of extract and lane 2 contained RNA after incubation with splicing extract. Clearly, the migration of the RNA was retarded after incubation in the splicing extract, indicating that it was bound by proteins. The pattern obtained in this experiment, i.e. that of two retarded complexes, was repeatedly seen under a variety of conditions, i.e. different concentrations of $MgCl_2$, presence or absence of ATP, presence or absence of a GpppG cap structure on the U5 snRNA, in the presence of varying amounts of competitor tRNA, and also when performed with sonicated yeast extract (data not shown). In an attempt to disrupt endogenous snRNP particles and, possibly, increase the concentration of free snRNP proteins available to bind exogenous snRNA, extracts were pre-treated with micrococcal nuclease, but this had no effect on the retardation pattern obtained (data not shown). When, for comparison, splicing extract was run on such a non-denaturing gel, blotted to a nylon membrane and probed with a U5-specific oligonucleotide probe, U5 snRNA was seen to be present in several complexes (Figure 5.2D). The fastest migrating of these, presumably the free U5 snRNP, co-migrated with the slowest migrating complex observed in the reconstitutuion experiment. It was possible, therefore, that this represented a reconstituted U5 snRNP particle.

To test whether the U5 snRNA was indeed being sequestered into snRNP complexes, immunoprecipitations were performed on reconstitution mixes using anti-8.4 antibodies and anti-Sm antibodies (from serum #24, M.Dalrymple, this laboratory), both of which would precipitate genuine U5 snRNP particles. The results of these experiments indicated that a tiny proportion (less than 1%) of the U5 snRNA was precipitable with anti-Sm antibodies after incubation in splicing extract, whereas none was precipitable with anti-8.4 antibodies (data not shown). This strongly suggests that the gel retardation of the U5 snRNA does not represent the formation of snRNP complexes, but rather is due to the non-specific RNA-binding activity of proteins in the extracts.

5.3 RNA-BINDING PROPERTIES OF THE PRP8 PROTEIN

Since the PRP8 protein is a component of the U5 snRNP and of the spliceosome, it is possible that it directly binds RNA, either the U5 snRNA or the substrate pre-mRNA, in these complexes. This was investigated using the technique of "North-Western blotting" whereby proteins are transferred to nitrocellulose membranes from SDS-polyacrylamide gels and probed for RNA-binding properties with radiolabelled RNA.

When total yeast splicing extract, or proteins precipitated with anti-8.4 antibodies, were probed with either pre-mRNA or U5 snRNA, no binding to a 280kD protein was detected (data not shown). This may suggest that the PRP8 protein does not bind RNA, but it is also possible that the amount of PRP8 protein present on such blots was too small to be detected by this method.

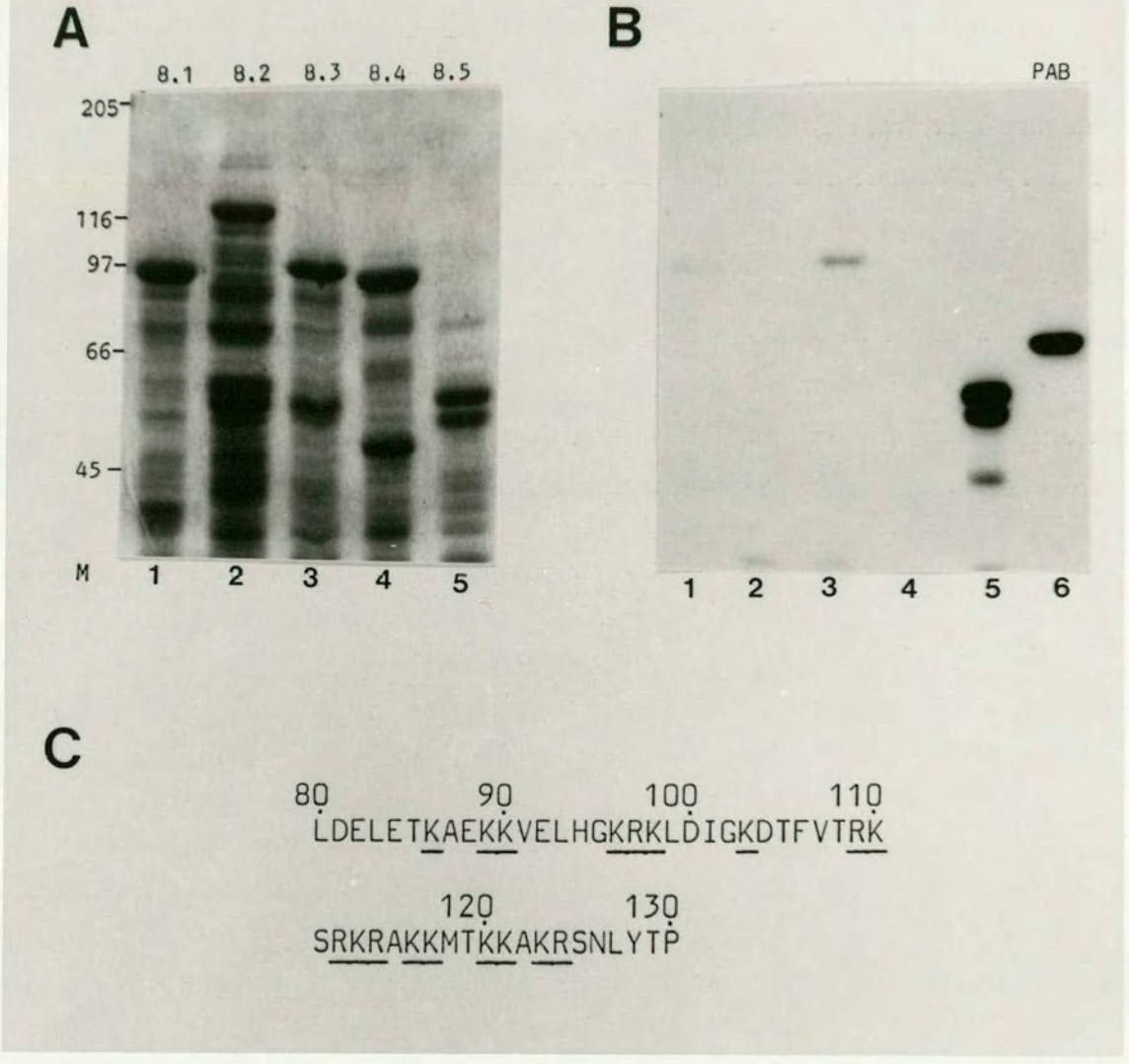
Fusion proteins, however, provide a ready source of plentiful amounts of particular regions of the PRP8 protein, so TRP8.1 to TRP8.5 (see Section 3.2.3) were tested for their ability to bind RNA and Figure 5.2 shows the result of such an experiment. Figure 5.2A depicts a Coomassie-stained SDS-polyacrylamide gel displaying insoluble E.coli protein preparations containing each of the five TrpE-PRP8 fusion proteins (lanes 1-5). Figure 5.2B depicts the result of probing a blot, corresponding to the gel in Figure 5.2A, plus purified poly(A)-binding protein in lane 6, with a polyadenylated pre-mRNA (rp51ApA, transcribed in vitro from pSPrp51ApA). As expected, the poly(A)-binding protein binds the transcript through an interaction with

FIGURE 5.2: TRP8.5 FUSION PROTEIN BINDS RNA

(A) Insoluble protein fractions from induced E.coli HB101 cells containing pTRP8.1-8.5. Samples from insoluble protein fractions were fractionated and analysed as described in Figure 3.3C: TRP8.1 (lane 1), TRP8.2 (lane 2), TRP8.3 (lane 3), TRP8.4 (lane 4), TRP8.5 (lane 5). Sizes of protein molecular weight markers (M) are indicated (kD).

(B) Blot assay for RNA binding. Samples of insoluble protein fractions containing TRP8.1-8.5 were subjected to electrophoresis as in (A), but with an additional track containing 2μg of purified polyadenylate-binding protein (lane 6). Proteins were electroblotted to nitrocellulose and probed with radiolabelled rp51ApA transcript.

(C) Amino acid sequence of a portion of the PRP8 protein represented in TRP8.5. Basic residues are indicated by underlining.


A
8.1
8.2
8.3
8.4
8.5
205
116
97
66
45
M
1
2
3
4
5
B
PAB
1
2
3
4
5
6
C
80
90
100
110
LDELETKAEKKVELHGKRKLDIGKDTFVTRK
120
130
SRKRAKKMTKKAKRSNLYTP

its poly(A) tail (lane 6) and, of the TrpE fusion proteins, only TRP8.5 binds the RNA (lane 5). The failure of TRP8.1-TRP8.4 to bind the transcript to any significant extent shows that the TrpE portion of the fusion proteins does not possess RNA-binding activity. The binding of RNA by TRP8.5 is therefore due to the PRP8 portion of the fusion protein.

To test whether this binding is specific for intron-containing pre-mRNA species, further experiments were performed in which TRP8.5 was probed with U5 snRNA and anti-sense transcript from exon 2 of the yeast CYH2 gene. In both cases, TRP8.5 bound to the RNA species as strongly as to the rp51ApA transcript, indicating that the binding activity of the region of the PRP8 protein present in TRP8.5 is not sequence-specific (data not shown).

5.4 DISCUSSION

In this chapter, it was shown that exogenous U5 snRNA was not reconstituted into snRNP particles when incubated in yeast whole cell extracts, as assayed by precipitability by antibodies which precipitate endogenous U5 snRNP particles. Many possible reasons for this can be envisaged, a few of which are outlined below.

In the reconstitution of the U1 snRNP (Hamm et al., 1987; Patton et al., 1987), extracts which are known to be rich in free snRNP proteins were used and this is probably not true of yeast splicing extract. Attempts to disrupt endogenous U5 snRNPs with micrococcal nuclease may have been unsuccessful on two counts; (i) although micrococcal nuclease digestion leads to degradation of full-length U5 snRNA in splicing extracts, smaller fragments remain uncleaved, presumably protected by proteins (data not shown), (ii) snRNP proteins may have been rendered insoluble on disruption of the complexes. It may be that the extracts used in the U1 snRNP studies contain more snRNP proteins than non-specific RNA-binding proteins. This is unlikely to be the case in yeast whole cell extracts, given the low abundance of snRNPs. Therefore, upon addition of the snRNA, there may be competition between the snRNP-specific proteins and non-specific RNA-binding proteins for binding, with the former predominating in Xenopus oocyte and HeLa S100 extracts and the latter predominating in yeast cell extracts. This would explain the sequestering of exogenous U5 snRNA into complexes

which are retarded on non-denaturing gels. It is not clear, though, why such binding would not be competed by the addition of an excess of tRNA.

The TRP8.5 fusion protein was found to possess a non-specific RNA-binding property. Although it is possible that the interface between the TrpE and the PRP8 portions of the fusion protein generate a novel RNA-binding site, this interaction is probably mediated by the PRP8 portion alone. The region of the PRP8 protein present in this fusion protein (Figure 5.3C) contains a segment between amino acids 85 and 124 in which 19 out of 39 are basic residues (S.Jackson, Ph.D. thesis, University of Edinburgh; S.Dunbar, personal communication). This represents a strong candidate for a RNA-binding site within the PRP8 component of TRP8.5. It has been shown that the PRP8 protein can be cross-linked, by UV-irradiation, to pre-mRNA in the spliceosome, which strongly implies a direct binding to the splicing substrate (E.Whittaker, personal communication). It is tempting to speculate, in this light, that the basic region between residues 85 and 124 constitutes the substrate-binding site.

There are several possible reasons why TRP8.5 does not show binding specificity for pre-mRNA. The PRP8 protein may not have a sequence-specific binding capacity, but rather may function to bind a non-conserved region of the substrate in the spliceosome, e.g. exon 1. Alternatively, the specificity of RNA-binding may be conferred by regions of the PRP8 protein not represented in TRP8.5, or, indeed, by other proteins in the U5 snRNP, or in the spliceosome.

The finding that PRP8 protein directly contacts the pre-mRNA, combined with its presence in the U5 snRNP, leads to speculation on its relationship to the intron-binding protein of HeLa cells (Gerke and Steitz, 1986; Tazi et al., 1986). This protein, of 70kD (Gerke and Steitz, 1986) or 100kD (Tazi et al., 1986), binds specifically to the polypyrimidine tract upstream of the 3' splice site and, under certain conditions, co-purifies with the U5 snRNP. The possibility that the PRP8 protein represents a yeast homologue of this protein is considered in Chapter 6.

CHAPTER 6

IDENTIFICATION OF A PRP8 HOMOLOGUE IN HELA CELLS

6.1 INTRODUCTION

In most respects, the process of pre-mRNA splicing in yeast cells closely resembles that in higher eukaryotic cells. As discussed in Chapter 1, the basic two-step pathway is conserved and, in both cases, this takes place in a large complex, the spliceosome. By non-denaturing gel electrophoresis, three splicing specific complexes can be separated in yeast and HeLa cell in vitro splicing reactions, and these are equivalent on the basis of snRNA and splicing substrate, intermediate and product content (see Section 1.7). In Chapter 4, it was shown that a U4/U5/U6 multi-snRNP complex forms in yeast extracts under similar conditions to those which promote formation of an equivalent complex in HeLa cell extracts.

In this light, it might be predicted that at least some of the protein splicing factors will be conserved between yeast and human cells, as, indeed, are the spliceosomal snRNAs to varying extents (see Section 1.5.2.1). Evidence for this is provided by the finding that certain patient anti-Sm sera can precipitate both human and yeast snRNPs, indicating conservation between the species of the Sm-epitope(s) on at least one protein in each snRNP (see Section 1.5.2.2). The generation of antibodies against the β-galactosidase-PRP8 fusion proteins provided an opportunity to investigate whether HeLa cells contain a homologue of the yeast PRP8 protein. However, snRNAs were not precipitated from HeLa cell nuclear extracts by antibodies against FP8.1-FP8.4 (M.Lossky; Ph.D. thesis, Imperial College, London).

6.2 IDENTIFICATION BY IMMUNOBLOTTING OF A HUMAN HOMOLOGUE OF PRP8

6.2.1 Antibodies Against PRP8 Detect a Large HeLa Protein

The inability of anti-PRP8 antibodies to precipitate snRNAs from HeLa cell extracts (see Section 6.1) does not preclude the existence of a human homologue of the PRP8 protein. It is possible, for instance, that the antibodies do not recognise the native form of such a homologue, or that the homologue is not stably associated with any snRNAs under the conditions tested. Assaying for the precipitation of snRNAs by anti-PRP8 antibodies did not, therefore, constitute a very rigorous test for the existence of a homologous protein in human cells.

HeLa cell nuclear extract (a gift from P. Sharp, M.I.T) was tested for the presence of proteins which are recognised by anti-PRP8 sera (anti-8.1 to anti-8.4) in immunoblotting experiments, and the results are shown in Figure 6.1A. Clearly, a large number of HeLa cell nuclear proteins cross-reacted with each of the anti-PRP8 sera and few firm conclusions could be drawn from such an experiment. However, it is interesting to note the detection of a species of approximately 220kD by anti-8.1 (lane 2), anti-8.2 (lane 3), and anti-8.4 (lane 4) sera, but not by anti-8.3 serum (lane 1). As an initial test for which of these signals is due to antibodies against the PRP8 portion of the fusion proteins, antibodies from anti-8.2 serum were affinity purified against β-galactosidase or FP8.2 and used to probe blots of HeLa cell nuclear extract (Figure 6.1B). As before, crude anti-8.2 serum detected a large number of proteins (lane 1), many of which were detected by antibodies purified against FP8.2 (lane 3). Antibodies purified against β-galactosidase gave the same pattern, but with the exception of the 220kD species (lane 2), clearly indicating that only the 220kD signal results from antibodies specific to the PRP8 portion of FP8.2. Similar results were obtained using affinity purified antibodies from anti-8.1 serum (data not shown).

To confirm that antibodies to three different regions of the PRP8 protein recognise a 220kD HeLa cell protein, immunoblotting experiments were repeated with antibodies affinity purified against the corresponding TrpE-PRP8 fusion proteins (Figure 6.1C). This experiment showed that affinity purified anti-8.1 (lane 1), anti-8.2 (lane 2), and anti-8.4 (lane 3) antibodies detect the 220kD species in HeLa cell nuclear extract.

FIGURE 6.1: DETECTION OF A 220kD HELA PROTEIN IMMUNOLOGICALLY RELATED TO PRP8

(A) Probing HeLa cell nuclear extract with whole antisera. Aliquots (6μl) of nuclear extract were fractionated in an 8.5% SDS-polyacrylamide gel. electroblotted to nitrocellulose and probed with antisera as follows; anti-8.3 (lane 1), anti-8.1 (lane 2), anti-8.2 (lane 3), anti-8.4 (lane 4). Sizes of protein molecular weight markers (M) are indicated (kD).

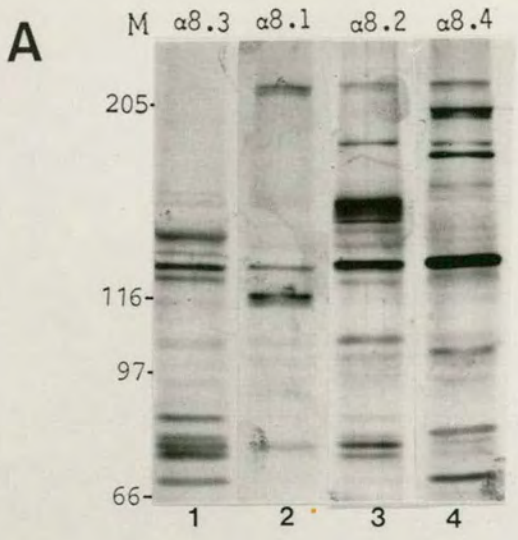
(B) Aliquots of HeLa cell nuclear extract were subjected to electrophoresis and electroblotted as in (A) and then probed as follows; anti-8.2 serum (lane 1), IgG from anti-8.2 serum affinity purified against β-galactosidase (lane 2), or against FP8.2 (lane 3). Markers as in (A).

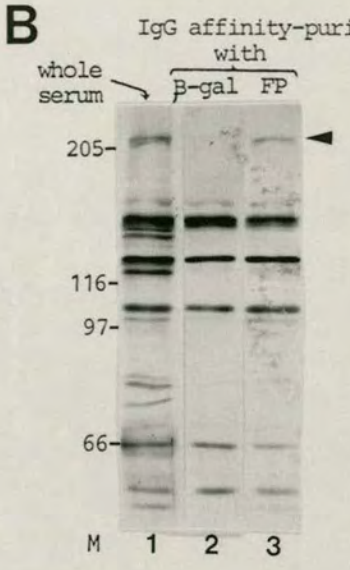
(C) Aliquots of HeLa cell nuclear extract were subjected to electrophoresis and electroblotted as in (A) and probed as follows; IgG from anti-8.1 serum affinity purified against TRP8.1 (lane 1), IgG from anti-8.2 serum affinity purified against TRP8.2 (lane 2), IgG from anti-8.4 serum affinity purified against TRP8.4 (lane 3). Markers as in (A).

FIGURE 6.2: PRP8-SPECIFIC ANTISERA DETECT A LARGE PROTEIN(S) IN A PURIFIED U5 snRNP FRACTION

Aliquots of 20S U5 snRNP fraction, purified as described in Bach et al. (1989), were fractionated in a 4-20% gradient SDS- polyacrylamide gel. The total protein content was visualised by staining with Coomassie Blue (lane 1). Protein from the remainder of the samples was electroblotted to nitrocellulose and probed as follows; pre-immune 8.4 serum (lane 2), immune anti-8.4 serum (lane 3), pre-immune 8.2 serum (lane 4), immune anti-8.2 (lane 5), IgG from anti-8.4 serum affinity purified against TRP8.4 (lane 6), IgG from anti-8.2 serum affinity purified against TRP8.2 (lane 7).

FIG 6.1


A
M α8.3 α8.1 α8.2 α8.4
205-
116-
97-
66-
1 2 3 4


B
IgG affinity-purified with
whole serum
β-gal FP
205-
116-
97-
66-
M 1 2 3

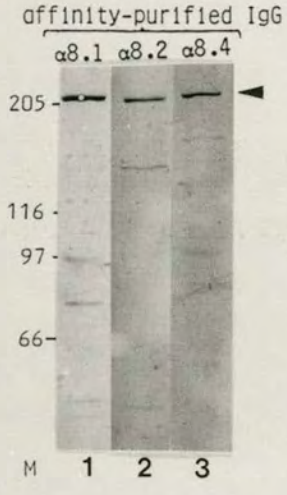
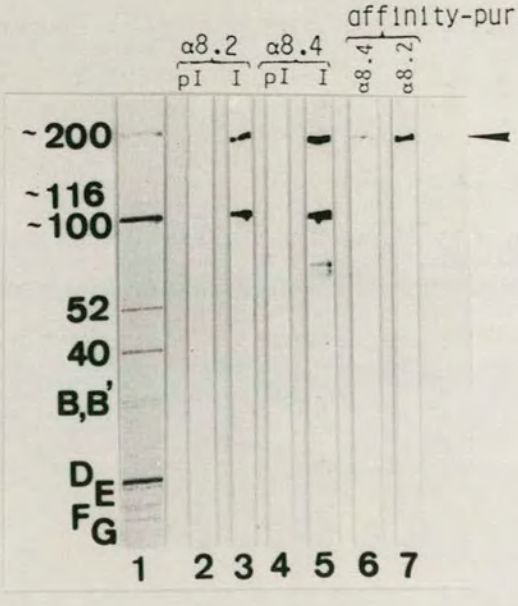

C
affinity-purified IgG
α8.1 α8.2 α8.4
205-
116-
97-
66-
M 1 2 3

FIG 6.2


affinity-purified
α8.2
pI I
α8.4
pI I
α8.4
α8.2
~200
~116
~100
52
40
B,B'
D
E
F
G
1 2 3 4 5 6 7

As for crude anti-8.3 serum, affinity purified anti-8.3 antibodies did not detect this species (data not shown). Control experiments confirmed that only antibodies affinity purified against the corresponding TrpE fusion protein detected the 220kD protein (data not shown).

6.2.2 The Human PRP8 Homologue Co-purifies with U5 snRNP Particles

It was shown that a proportion of purified U5 snRNP particles from HeLa cell extracts contain several specific proteins, two of which are larger than 200kD (Bach et al., submitted). To determine if either of these proteins corresponds to the species detected by anti-PRP8 antibodies (see Section 6.2.1), purified U5 snRNP proteins were separated on an SDS-polyacrylamide gel, blotted and probed with anti-PRP8 sera and antibodies (performed by R. Lührmann and shown in Figure 6.2). Lane 1 shows the range of U5 snRNP proteins revealed by Coomassie Blue staining, with the common core snRNP proteins B', B, D, D', E, F, and G and the U5 snRNP-specific proteins with apparent molecular weights of 40, 52, 100, 102, 116, and >200 (doublet) kD. When probed with anti-8.2 (lane 3) or anti-8.4 (lane 5) total sera, signals corresponding to the doublets at >200kD and approximately 100kD were obtained, but with anti-8.2 (lane 7) and anti-8.4 (lane 6) antibodies affinity purified against the corresponding TrpE fusion proteins, only a signal corresponding to a protein of >200kD was observed. This provides strong evidence that the human homologue of the yeast U5 snRNP-specific PRP8 protein is associated with a proportion of the U5 snRNP particles.

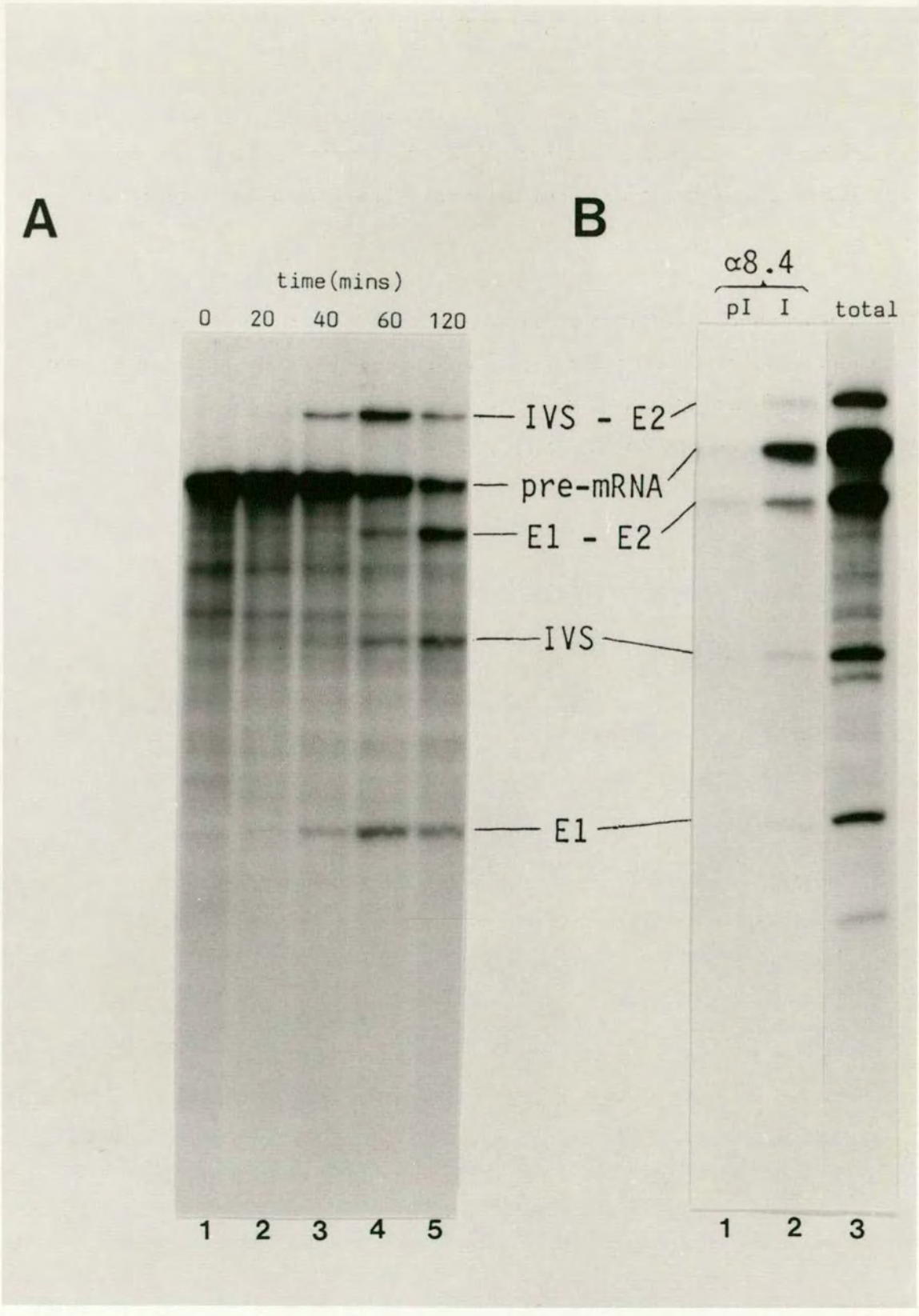
6.3 IMMUNOPRECIPITATION FROM HELA IN VITRO SPLICING REACTIONS

The presence of the PRP8 protein in the yeast spliceosome was demonstrated by the ability of anti-PRP8 antibodies to precipitate complexes containing splicing intermediates from an in vitro splicing reaction (M. Lossky, Ph.D. thesis; Imperial College, London). In order to test for the presence of the homologous protein in the human spliceosome, immunoprecipitation reactions were performed on in vitro splicing reactions in the HeLa cell nuclear extract. Figure 6.3A shows the time course of splicing using a SP6 RNA polymerase-transcribed RNA substrate containing the first two exons and IVS1 of the human β-globin gene (Krainer et al., 1984). The RNA

FIGURE 6.3: IMMUNOPRECIPITATION OF HELA SPLICEOSOME

(A) Time course of in vitro splicing reaction in a HeLa cell nuclear extract. Splicing of radiolabelled β-globin transcript was allowed to proceed at 30°C for the times indicated before RNA was purified and fractionated in a 10% polyacrylamide/7M urea gel.

(B) Immunoprecipitation from HeLa splicing reaction. Reactions were allowed to proceed for 60 minutes before being subjected to immunoprecioitation with pre-immune 8.4 (lane 1) or immune anti-8.4 (lane 2) antibodies. RNA was purified from immune complexes and analysed as in (A). Total RNA was purified from a half volume splicing reaction (lane 3).


A
time(mins)
0
20
40
60
120
IVS - E2
pre-mRNA
E1 - E2
IVS
E1
1
2
3
4
5
B
α8.4
pI
I
total
1
2
3

intermediates and products were identified on the basis of their electrophoretic mobilities relative to DNA size markers (not shown) and of the kinetics of their appearance and disappearance. It was clear that the splicing intermediates, free exon 1 and lariat intron-exon 2, were most abundant after 1 hour of incubation (compare lane 4 with others), suggesting that functional spliceosomes were most abundant at this time point. Accordingly, subsequent immunoprecipitation reactions were performed on reactions halted after 1 hour.

Immunoprecipitation reactions from such splicing reactions revealed that anti-8.1. anti-8.2 and anti-8.4 antibodies were all capable of precipitating pre-mRNA and all intermediate and product species (anti-8.3 antibodies were not tested). A typical example of such an experiment is presented in Figure 6.3B for anti-8.4 antibodies where antibodies from immune serum (lane 2) clearly precipitated more of the RNA species than did antibodies from pre-immune serum (lane 1). Precipitation of the spliced mRNA product was unexpected, since this species dissociates from the complex containing the lariat intron product (Konarska and Sharp, 1987) and, in addition, is not precipitated from yeast splicing reactions by anti-PRP8 antibodies (M.Lossky, Ph.D. thesis; Imperial College, London).

However, when the precipitation reactions were performed with antibodies affinity purified against TrpE fusion proteins, the RNA species were not precipitated to any significant extent (data not shown). In addition, the precipitation of the RNA species by anti-8.1 and anti-8.4 antibodies from total serum was blocked by competition with an excess of any β-galactosidase fusion protein, while that by anti-8.2 antibodies was unaffected, even by the corresponding fusion protein (data not shown). These data clearly indicate that the precipitation of the RNA species from splicing reactions was not specific to antibodies against the PRP8 portions of the β-galactosidase fusion proteins. The precipitation by anti-8.1 and anti-8.4 appears to be due to antibodies against the β-galactosidase portion of the fusion proteins, while that by anti-8.2 appears to be due to some other antibody specificity in the serum.

6.4 THE HUMAN HOMOLOGUE OF PRP8 IS Sm-PRECIPITABLE

The findings that anti-PRP8 antibodies failed to precipitate snRNAs from HeLa cell extracts (M.Lossky, Ph.D. thesis; Imperial College, London) or spliceosomes from

HeLa in vitro splicing reactions (see Section 6.3) may, initially, appear inconsistent with the discovery that the PRP8 homologue co-purifies with the human U5 snRNP. However, it is possible that the anti-PRP8 antibodies fail to recognise the homologue in its native form and so cannot precipitate it, or, therefore, any associated species, from nuclear extract. The ability of these antibodies to detect the homologue on an immunoblot provided the means to investigate the precipitability of this protein by anti-PRP8 antibodies and by anti-Sm and anti-m^3G antibodies.

It was shown that anti-PRP8 antibodies were unable to precipitate the homologous human protein of approximately 220kD from HeLa cell nuclear extract, as assayed by probing blots of the precipitated material with anti-8.4 serum (data not shown). To test for the ability of anti-Sm and anti-m3G antibodies (both of which precipitate all the spliceosomal snRNPs) to precipitate this species, experiments were performed using nuclear extract which had been pre-incubated for 45 minutes under "mock splicing" conditions in the presence or absence of ATP. The results, shown in Figure 6.4A, indicate that anti-8.4 antibodies detect a species of greater than 200kD precipitated strongly by anti-Sm antibodies in the presence of ATP (lane **6**), but poorly in its absence (lane **5**). Two species, the larger of which co-migrates with the strongly Sm-precipitable species, are weakly precipitated by anti-m^3G antibodies (lane 2), with a slight increase observed upon incubation in the presence of ATP (lane 3). Anti-m^3G precipitation of this doublet proved to be quite variable, although always very weak, lane 7 showing an example of their precipitation from an extract which had not been pre-incubated. A weak signal corresponding to a protein co-migrating with the smaller of the two anti-m^3G precipitable species was observed in the anti-Sm precipitate (lane 5), but was not enhanced by ATP incubation (lane 6), nor was it seen in subsequent precipitations (see Figures 6.4B and 6.4C).

To confirm that the strongly Sm-precipitable species represents the PRP8 homologue detected in total nuclear extract in Section 6.2.1, Sm-precipitated material was blotted and probed with anti-8.1 to anti-8.4 sera. The experiment (Figure 6.4B) gave the same pattern of detection as was obtained for the homologue of PRP8 identified in Section 6.2.1, i.e. anti-8.1 (lane 2), anti-8.2 (lane 3) and anti-8.4 (lane 4) sera detected the large protein while anti-8.3 (lane 1) serum did not. This strongly suggests that the Sm-precipitable protein is, indeed, the human homologue of PRP8.

FIGURE 6.4: THE HUMAN HOMOLOGUE OF PRP8 IS Sm-PRECIPITABLE

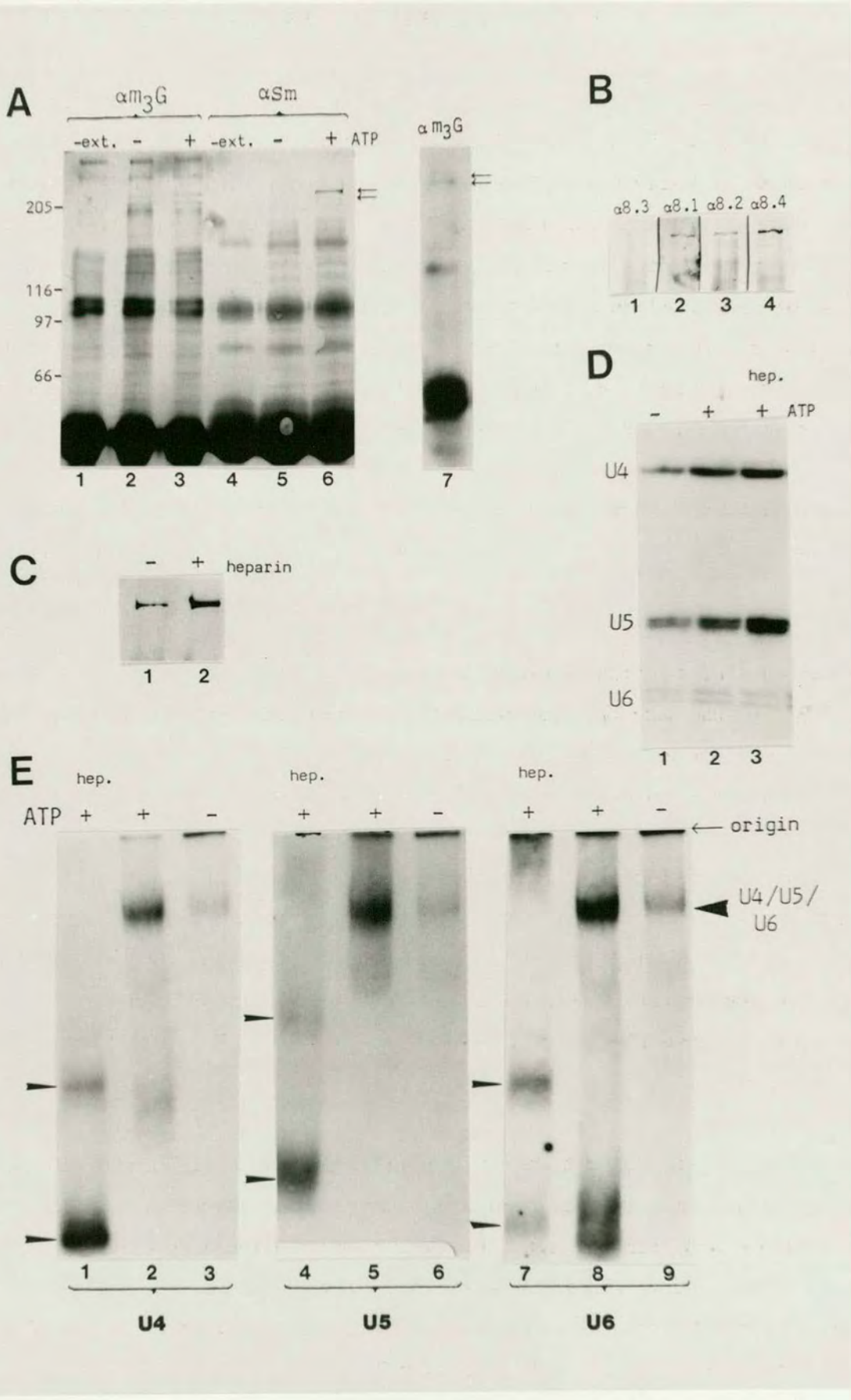
(A) Precipitation from mock splicing reactions in HeLa cell nuclear extracts. Extract was incubated at 30°C for 45 minutes in splicing buffer in the absence of ATP (lanes 2 and 5) or in the presence of 1.6mM ATP and 8mM creatine phosphate (lanes 3 and 6) and subjected to immunoprecipitation with anti-m^3G (lanes 2 and 3) or anti-Sm (lanes 5 and 6) antibodies. Precipitated material was fractionated in an 8.5% SDS-polyacrylamide gel, blotted to nitrocellulose and probed with anti-8.4 serum. Mock immunoprecipitations were performed with anti-m^3G (lane 1) and anti-Sm (lane 4) antibodies. Sizes of protein molecular weight markers (M) are indicated (kD). Lane 7 shows a separate anti-m^3G immunoprecipitation performed from a nuclear extract which was not pre-incubated.

(B) HeLa cell nuclear extract was incubated in the presence of ATP, subjected to anti-Sm immunoprecipitation, fractionated and blotted as in (A). The lanes were probed with serum as follows; anti-8.3 (lane 1), anti-8.1 (lane 2), anti-8.2 (lane 3), anti-8.4 (lane 4).

(C) HeLa cell nuclear extract was incubated in the presence of ATP, as in (A). Distilled H_2O (1/10th volume, lane 1) or heparin (1/10th volume of 50mg/ml, lane 2) was added and incubation continued at 30°C for 5 minutes. The reaction mixes were subjected to anti-Sm immunoprecipitation, fractionated and blotted as in (A) and probed with anti-8.4 serum.

(D) HeLa cell nuclear extract was incubated in the absence of ATP (lane 1) or in its presence (lanes 2 and 3) as in (A). Distilled H_2O (lanes 1 and 2) or heparin (lane 3) was added, as in (C), and the reactions subjected to anti-Sm immunoprecipitation. RNA was purified from immune complexes, fractionated in a 6% polyacrylamide/7M urea gel, blotted to Hybond N membrane and probed with 5'-end labelled oligonucleotides specific for human U4, U5 and U6 snRNAs.

(E) HeLa cell nuclear extract was incubated in the absence (lane 3) or presence of ATP (lanes 1 and 2) and dH_2O (lanes 2 and 3) or heparin (lane 1) was added, as in (C). The reaction mixes were then subjected to electrophoresis through a 4% non-denaturing polyacrylamide gel and were blotted to Hybond N membrane. The blot was sequentially probed with 5'-end labelled oligonucleotides specific for human U4 snRNA (lanes 1, 2 and 3), U5 snRNA (lanes 4, 5 and 6) and U6 snRNA (lanes 7, 8 and 9).


A
αm3G
αSm
-ext. - + -ext. - + ATP
205-
116-
97-
66-
1 2 3 4 5 6
αm3G
7
B
α8.3 α8.1 α8.2 α8.4
1 2 3 4
C
- + heparin
1 2
D
hep.
- + + ATP
U4
U5
U6
1 2 3
E
hep. hep. hep.
ATP + + - + + - + + -
origin
U4/U5/U6
1 2 3 4 5 6 7 8 9
U4 U5 U6

This result does not distinguish between the possibilities that the homologue of PRP8 is an Sm-antigen and is being directly recognised by the antibodies or that it is being precipitated through an interaction with Sm antigens, e.g. as a snRNP component. It was shown that the Sm-serum used in these precipitation reactions ("Kung") did not detect the homologue of PRP8 in an immunoblotting experiment (data not shown). This does not preclude the possibility, however, that the anti-Sm antibodies can recognise the homologue in its native form in the extract, but not in a denatured form on a blot.

Incubation of a yeast splicing extract in the presence of heparin had a profound effect on the snRNP population, seeming to disrupt snRNP-snRNP interactions and to partially dissociate individual snRNP particles (see Section 4.4). Following the ATP incubation, the effect of a brief incubation in the presence of heparin on the Sm-precipitation of the homologue of PRP8 was tested and the result is shown in Figure 6.4C. The presence of heparin resulted in an increase in the precipitation of the homologue of the PRP8 protein by Sm-antibodies (lane 2) compared to the amount precipitated when heparin was absent (lane 1). Figure 6.4D shows a blot of the RNA precipitated by the Sm-antibodies after incubation in the presence of ATP (lane 2) and in the presence of ATP and heparin (lane 3). The blot was probed with radiolabelled oligonucleotides specific for human U4, U5 and U6 snRNAs. The amounts of U5 and U6 snRNAs precipitated under these conditions parallels the amounts of the homologue of PRP8 precipitated, i.e. heparin increased the precipitation several-fold.

The effect on snRNP complexes of incubation of nuclear extract in the presence of heparin and ATP was analysed by electrophoresis through a non-denaturing gel, blotting and probing, sequentially, for U4, U5, and U6 snRNAs (Figure 6.4E). The effect of incubation in the presence of ATP (lanes 2, 5 and 8), as compared to incubation in its absence (lanes 3, 6 and 9), is essentially the same as previously described, i.e. material moves out of the well, U4/U5/U6 complex accumulates, as do free U4 and U6 particles (Konarska and Sharp, 1987; Konarska and Sharp, 1988). Incubation with heparin, following the ATP incubation, (lanes 1, 4 and 7) results in dissociation of the U4/U5/U6 complex and the appearance of several faster migrating particles, presumably due to partial dissociation of the snRNP complexes. These faster migrating particles are rendered more Sm-precipitable and, if the homologue of PRP8

remains associated with the U5 snRNP, this would explain its increased precipitability under these conditions.

If incubation in the presence of ATP, or in the presence of ATP and heparin, increases the precipitability of snRNP particles, it is possible that anti-PRP8 antibodies might precipitate the PRP8 homologue from a similarly treated extract. However, this species was not detectably precipitated by anti-8.1 or anti-8.4 antibodies under these conditions (data not shown).

If the homologue is being precipitated by Sm-antibodies through an interaction with the U5 snRNP or, indeed, through an association with the U4/U5/U6 multi-snRNP complex formed in HeLa cell nuclear extracts in the presence of ATP (Konarska and Sharp, 1988; Black, personal communication), it might be possible to diminish this interaction, and hence the precipitation, by disruption of the U4, U5, or U6 snRNAs. Disruption was achieved by incubation of nuclear extract with oligonucleotides complementary to regions of the snRNAs known to be available for base-pairing (Black, personal communication) and with RNase H, which digests DNA-RNA hybrids. Figure 6.5A shows a blot of total RNA in the nuclear extract following RNase H digestion in the presence of a control oligonucleotide (lane 1) or in the presence of oligonucleotides specific for U4 (lane 2), U5 (lane 3), or U6 (lane 4) snRNAs, and probed with radiolabelled probes for these snRNAs. Under such conditions, U4 and U6 snRNAs are essentially completely degraded, while U5 snRNA is approximately 50% degraded, consistent with the results of Black (personal communication). Figure 6.5B shows the result of probing Sm-precipitates from such RNase H-treated extracts for the homologue of PRP8, and it is seen that degradation of U4, U5, or U6 snRNAs has little effect on the amount of this protein precipitated.

6.5 RNA-BINDING PROPERTIES OF THE HUMAN HOMOLOGUE OF PRP8

A human protein which specifically binds the 3' splice site region of pre-mRNA was found to be associated with U5 snRNP particles and was reported as being of apparent molecular weight 70kD (Gerke and Steitz, 1986) or 100kD (Tazi et al., 1986). The apparent conflict in the reported size of this protein was, presumably, the result of proteolytic degradation during fractionation of the cell extracts. The possibility existed that both these species may represent proteolytic products of the PRP8 homologue, so

FIGURE 6.5: Sm-PRECIPITATION OF THE PRP8 HOMOLOGUE IS UNAFFECTED BY CLEAVAGE OF U4, U5 OR U6 snRNAs.

(A) HeLa cell nuclear extract was incubated in the presence of ATP, as in Figure 4.1A, but with the addition of 2 units of RNase H and 1μg of oligonucleotide as follows; control (lane 1), U4 snRNA-specific (lane 2), U5 snRNA-specific (lane 3), U6 snRNA-specific (lane 4). Total RNA was purified, fractionated in a 6% polyacrylamide/7M urea gel, blotted to Hybond N membrane and probed with 5'-end labelled oligonucleotides specific for human U4, U5 and U6 snRNAs.

(B) Nuclear extract was treated as in (A), but then subjected to immunoprecipitation with anti-Sm antibodies. Precipitated material was fractionated in an 8.5% SDS-polyacrylamide gel, blotted to nitrocellulose and probed with anti-8.4 serum. Lanes as in (A).

FIGURE 6.6: THE HOMOLOGUE OF PRP8 BINDS RNA

(A) HeLa cell nuclear extract was fractionated in an 8.5% SDS- polyacrylamide gel, blotted to nitrocellulose and probed with radiolabelled β-globin transcript. Sizes of protein molecular weight markers (M) are indicated (kD).

(B) HeLa cell nuclear extract was incubated in the presence of ATP and heparin was added, as in Figure 6.4C. The reaction was subjected to immunoprecipitation with anti-Sm antibodies and precipitated material was fractionated in an 8.5% SDS- polyacrylamide gel and blotted to nitrocellulose. Duplicate lanes were probed with anti-8.4 serum (lane 1) and radiolabelled β-globin transcript (lane 2). Markers as in (A).

FIGURE 6.7: DETECTION OF AN IMMUNOLOGICALLY RELATED PROTEIN IN <u>DROSOPHILA</u> WHOLE CELL EXTRACT

(A) <u>Drosophila</u> whole cell extract was fractionated in an 8.5% SDS-polyacrylamide gel, blotted to nitrocellulose and probed with sera as follows; anti-8.3 (lane 1), anti-8.1 (lane 2), anti-8.2 (lane 3), anti-8.4 (lane 4). Sizes of protein molecular weight markers (M) are indicated (kD).

(B) HeLa cell nuclear extract (lane 1) and <u>Drosophila</u> whole cell extract (lane 2) was fractionated and blotted as in (A) and probed with IgG from anti-8.1 serum affinity purified against FP8.1. Markers as in (A).

FIG 6.5

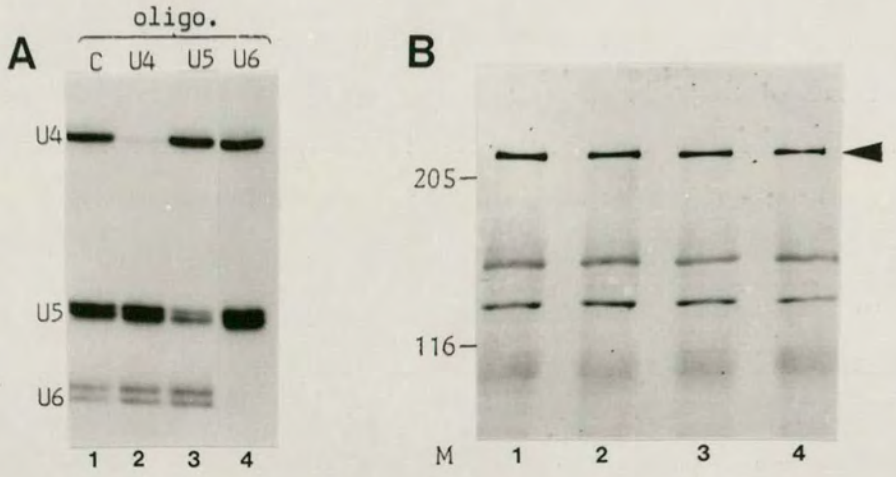


FIG 6.6

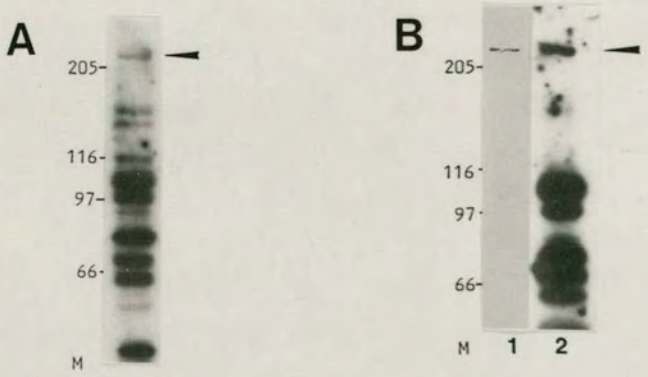
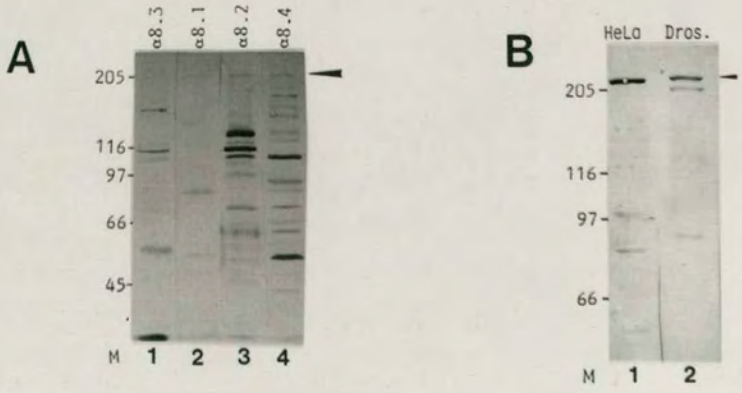


FIG 6.7



the RNA-binding properties of the homologue were investigated by probing protein blots with radiolabelled transcripts.

Initially, total protein in HeLa cell nuclear extract was fractionated on a SDS-polyacrylamide gel, blotted to nitrocellulose and probed with radiolabelled human β-globin transcript containing exon 1, IVS1 and exon 2 (Figure 6.6A). This revealed that there is, indeed, a large protein which binds pre-mRNA. In similar experiments, where parallel strips were probed with pre-mRNA and anti-8.4 antibodies, this protein was shown to co-migrate with the homologue of PRP8 (data not shown).

The criterion of co-migration on an SDS-polyacrylamide gel was insufficient to identify the RNA-binding protein as the homologue of PRP8, so the Sm-precipitability of the RNA-binding protein was assayed (Figure 6.6B). HeLa cell nuclear extract was pre-incubated under "mock splicing" conditions in the presence of ATP, heparin was added and incubation continued for 5 minutes before being subjected to immunoprecipitation with anti-Sm antibodies. The precipitated material was fractionated by SDS-PAGE and assayed for RNA-binding or probed for the presence of the homologue of PRP8 as before. Several proteins with RNA-binding capacity were precipitated by the Sm-antibodies (lane 2), the largest of which co-migrated with the PRP8 homologue detected with anti-8.4 serum (lane 1). This strongly suggests that the protein of approximately 220kD, which binds pre-mRNA in this assay, is the human homologue of PRP8.

If this protein is related to the intron-binding protein, then its binding of RNA on blots should be specific to intron-containing transcripts (Gerke and Steitz, 1986; Tazi et al., 1986). However, the human homologue of PRP8 was shown to bind, with equal efficiency, a truncated β-globin transcript which lacked any intron sequence (data not shown). The RNA-binding properties of this protein are not intron sequence-specific, as assayed on protein blots, and it appears, therefore, to be unrelated to the intron-binding protein of the U5 snRNP particle.

6.6 IDENTIFICATION BY IMMUNOBLOTTING OF A PUTATIVE *DROSOPHILA MELANOGASTER* HOMOLOGUE OF PRP8

Total protein extract of *Drosophila melanogaster* cultured cells was tested for the presence of species recognised by antisera to FP8.1-FP8.4 by immunoblotting. The

results, shown in Figure 6.7A, revealed a similar pattern to that obtained with HeLa cell nuclear extract (see Section 6.2.1), in that many proteins were detected by each antiserum and a species with an apparent molecular weight of greater than 200kD was detected by anti-8.1 (lane 2), anti-8.2 (lane 3) and anti-8.4 (lane 4) sera, but not by anti-8.3 (lane 1) serum. An immunoblot of D.melanogaster cell extract probed with affinity purified anti-8.1 antibodies (Figure 6.7B, lane 2) showed that the large protein was the most strongly detected and migrated slightly slower than the human homologue (lane 1). A species with apparent molecular weight of approximately 200kD, also detected by affinity purified anti-8.1 antibodies, may represent a degradation product of the larger species within this extract. At the moment, no further experimental evidence exists in support of this protein representing a true D.melanogaster homologue of the yeast PRP8 protein.

6.7 DISCUSSION

In this chapter, it has been demonstrated that HeLa cells contain a protein which is recognised on immunoblots by anti-PRP8 antibodies. It has an apparent molecular weight some 60kD smaller than the yeast PRP8 protein and it is not detected by anti-8.3 antibodies, perhaps suggesting that it lacks a domain homologous to that represented in FP8.3. The homologous protein is not, however, precipitable by antibodies against any of FP8.1 to FP8.4, and this could be due to one of several reasons. It may, for instance, possess only small regions of structural similarity which are inaccessible to antibody binding in its native form, either due to folding of the protein or to its interaction with other molecules. It is, of course, possible that the HeLa protein has strong similarity to the PRP8 protein, but that it is completely inaccessible to antibody binding within a large complex.

It was shown that the putative homologue of PRP8 is one of a number of proteins which co-purify with human U5 snRNP particles (Bach et al., 1989). This strongly supports the idea that this protein is functionally homologous to the yeast PRP8 protein, since they are both associated with the U5 snRNP particle. There would appear to be a difference in the stability of the interaction with the snRNP particle between the yeast and human proteins. Only 15-20% of purified human U5 snRNPs contain the homologue of PRP8, or any U5-specific proteins, in the presence of low

$Mg2^+$ concentrations, while high concentrations of this divalent cation completely abolish interactions of the specific proteins with the core snRNP particle (Bach et al., 1989). In yeast, on the other hand, the PRP8-U5 snRNP interaction is stable over a wide range of concentrations of monovalent and divalent cations (M. Lossky, in Lossky et al., 1987).

That the PRP8 homologue is precipitable by Sm-antibodies indicates either that the protein carries an Sm-epitope(s) and is being directly recognised by the antibodies, or that it is associated with other Sm-reactive proteins. The protein is not detected on immunoblots by Sm-antisera implying that it does not carry any Sm-epitopes, although it is possible that such an epitope is not recognised in the denatured form. The finding that Sm-precipitation is dependent on incubation of the extract in the presence of ATP suggests that the protein, if being directly recognised by the antibodies, is becoming available for precipitation or that it is becoming associated de novo with Sm-reactive proteins, presumably in a snRNP particle. Precipitation of U4, U5 and U6 snRNAs with Sm-antibodies is increased by pre-incubation in the presence of ATP (Figure 6.4D), but not to the extent which would explain the increased precipitation of the PRP8 homologue, therefore the ATP effect cannot be explained simply by an increased precipitation of snRNP particles containing the homologous protein. It is possible that some, or all, of the U5 snRNP-specific proteins cycle on and off the core particle, thus explaining the small proportion of purified particles which contain them (Bach et al., 1989) and that upon pre-incubation in the presence of ATP, more of the proteins cycle on to the U5 snRNP particle. Alternatively, it could be that the U5 snRNPs which become available de novo during ATP incubation are structurally distinct from those already available in that they contain the homologue of PRP8.

The reasons for the poor, and variable, anti-m^3G precipitation of the homologue, and the additional precipitation of a slightly smaller species, are not clear. Immunoaffinity purification over an anti-m^3G column constitutes the first stage of the U5 snRNP purification (Bach et al., 1989), after which a proportion of U5 snRNPs still contain the PRP8 homologue (see Section 6.2.2). It may be that, under the conditions used for the immunoprecipitations in this study, binding of the cap by anti-m^3G antibodies induces a conformational change which causes the PRP8 homologue to dissociate from most of the snRNP particles. Perhaps, due to such a conformational

change, the PRP8 homologue which remains associated is rendered susceptible to limited proteolysis, thus explaining the precipitation of the smaller species.

Oligonucleotide-directed cleavage of U4 and U6 snRNAs has been shown to abolish the formation of an ATP-dependent U4/U5/U6 snRNP complex (Black, personal communication). The failure of such cleavage to diminish the Sm-precipitability of the homologue of PRP8 shows that this protein does not become associated with snRNPs solely during formation of the U4/U5/U6 complex. Cleavage of 50% of U5 snRNA has no effect on the Sm-precipitability of the homologue. This could be due to (i) precipitation of the homologue not being mediated by the U5 snRNP, (ii) snRNP structure not being disrupted by cleavage at this region of the snRNA, or (iii) the snRNA in snRNPs associated with the homologue being inaccessible to the oligonucleotide and RNase H (i.e. the 50% uncleaved U5 snRNA).

A protein with apparent molecular weight in excess of 200kD has been identified in the human spliceosome and has been shown to become cross-linked to pre-mRNA substrate upon UV-irradiation (M.Garcia-Blanco, M.I.T.; personal communication). That the yeast PRP8 protein can also be cross-linked to the substrate pre-mRNA (E.Whittaker, personal communication) strongly suggests that this protein represents the homologue of PRP8 and that it, too, has a RNA-binding role in the spliceosome. Interestingly, attempts to immunoprecipitate this protein with anti-PRP8 antibodies gave similar results to those obtained when attempting to precipitate spliceosome complexes (see Section 6.3), i.e. the cross-linked protein was precipitated by immune antibodies, but this effect was not specific to antibodies against the PRP8 portion of the fusion proteins (M. Garcia-Blanco, M.I.T.; personal communication)

The RNA-binding studies in this chapter suggest that the PRP8 homologue does not bind a specific sequence in the pre-mRNA. This indicates that the protein is distinct from the intron-binding protein (Gerke and Steitz, 1986; Tazi et al., 1986) and supports the finding that none of the smaller U5 snRNP-specific proteins are degradation products of the larger species (Bach et al., 1989). These results add significance to the findings, presented in Chapter 5, that the region of the yeast PRP8 protein present in TRP8.5 has a non-specific RNA-binding capacity and suggest a role for the yeast and human proteins in binding to non-conserved regions of the substrate in the spliceosome. Gerke and Steitz (1986) detected a protein of apparent molecular weight greater than 200kD which binds RNA, as assayed on blots, and which co-purifies

with snRNPs. These properties, combined with the fact that it did not display an intron-specific pattern of RNA-binding, suggest that this protein is the human homologue of PRP8.

The immunoblotting experiments using Drosophila melanogaster whole cell extracts show that there is a protein in this species which displays a similar pattern of immunological cross-reactivity with the yeast PRP8 protein as does the human homologue. However, definitive identification of this protein as a true functional homologue will require evidence that it is a component of the U5 snRNP particle.

CHAPTER 7

FINAL DISCUSSION

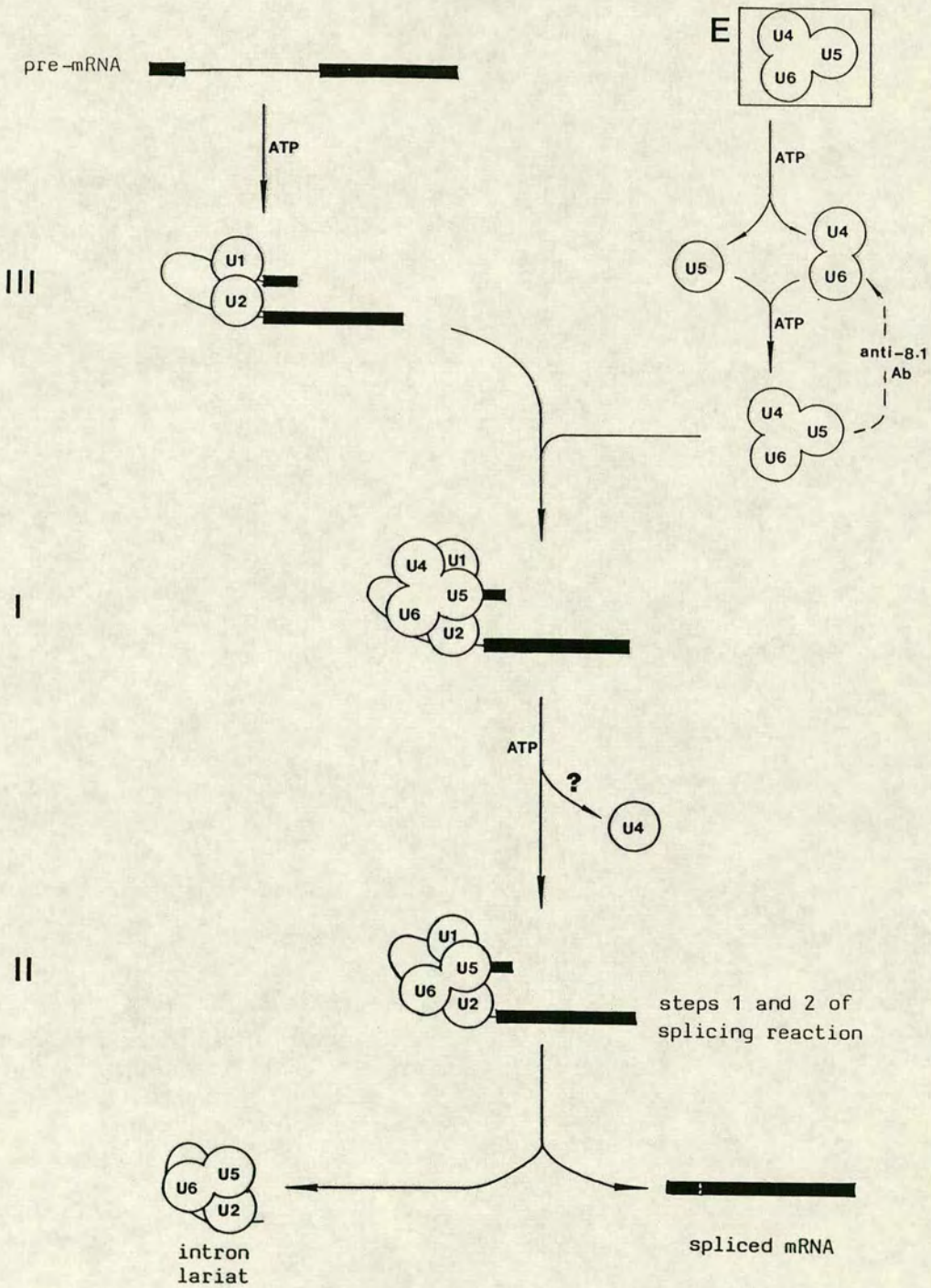
7.1 PRP8 AND PRE-mRNA SPLICING

The results described in this thesis can be used, in conjunction with published data, to postulate a model for spliceosome formation (Figure 7.1). The PRP8 protein is a stable component of U5 snRNP particles, a variable proportion of which exist, in splicing extracts, in endogenous snRNP complexes. Upon incubation in the presence of ATP, this complex dissociates to give free U5 and U4/U6 snRNP particles which can then assemble into "active" U4/U5/U6 snRNP complexes, structurally distinct from the endogenous complexes. This ATP-dependent complex then joins the early splicing complex III to convert it to complex I, constituting a major step in spliceosome formation. The PRP8 protein is an integral component of the functional spliceosome, where it directly contacts the pre-mRNA, during the splicing reaction. Following step 2 of the reaction, the spliceosome dissociates, spliced mRNA is released and the intron lariat remains associated with snRNPs, including PRP8-containing U5 snRNP. Therefore, the PRP8 protein is a component of at least four different types of complex through the course of spliceosome assembly and disassembly; (i) the U5 snRNP, (ii) the ATP- dependent U4/U5/U6 snRNP complex, (iii) the active spliceosome, and (iv) the post-splicing, intron-containing complex.

As yet, there are few clues as to the function of PRP8 in pre-mRNA splicing. The role of the U5 snRNP in mammalian splicing is unknown, since suggestions that it is involved very early in 3' splice site selection seem to be contradicted by its absence from early splicing complexes. It may be that the U5 snRNP acts to deliver the 3' splice site-specific intron-binding protein to the pre-mRNA but does not, itself, stably join the splicing complex until later in the pathway of formation. In yeast, the 3' splice site is not important for complex formation, so there may not be a homologue of the intron-binding protein. Thus, the role of the yeast U5 snRNP remains completely unknown.

FIGURE 7.1: MODEL OF PRE-mRNA SPLICING REACTION

Model postulated on the basis of a combination of published data and results presented in this thesis.


pre-mRNA
ATP
E
U4
U5
U6
ATP
III
U1
U2
U5
U4
U6
ATP
anti-8.1
Ab
U4
U5
U6
U4
U1
U5
U6
U2
I
ATP
?
U4
U1
U5
U6
U2
II
steps 1 and 2 of
splicing reaction
U6
U5
U2
intron
lariat
spliced mRNA

The immunological approach which has permitted the identification of the various PRP8-containing complexes will, in combination with genetic approaches, be instrumental in their further analysis and in the investigation of the specific function of PRP8. I will present a brief outline of the lines of investigation which may prove most successful, some of which have already been embarked upon in this laboratory.

Purification of the stable U5 snRNP particle by immunoaffinity chromatography would be very useful. In this way, the other protein components of the particle could be identified and the Sm-antigen(s) determined and, possibly, sufficient protein sequence information could be obtained to allow synthesis of oligonucleotide probes and, hence, cloning of the genes encoding them. The raising of antibodies to fusion proteins, exactly as described in this thesis for PRP8, would greatly expand the bank of immunological probes available to study the role of the U5 snRNP in the splicing process. Purified U5 snRNPs, if disrupted, may provide a source of free snRNP proteins to achieve successful reconstitution of the particle using radiolabelled snRNA. The possibility that sequence specificity of RNA-binding is conferred upon PRP8 by the structure of the snRNP could be investigated with purified particles.

As mentioned in Section 5.4, the anti-PRP8 antibodies have led to the demonstration that the PRP8 protein can be directly cross-linked to pre-mRNA in the spliceosome, suggesting direct contact (E. Whittaker, personal communication). Identification of the RNase T1 fragment which becomes cross-linked to the protein will define the PRP8 binding site and give clues as to its function in the spliceosome. Comparison of the binding sites in different pre-mRNAs will indicate whether PRP8 possesses a sequence preference for RNA-binding.

Affinity purified antibodies against the various regions of PRP8 may have different effects on complex formation if present during the splicing or mock-splicing reaction. In this way, it may be possible to define the roles played by different regions of this huge protein in interactions with other components of the splicing machinery. In a similar vein, an analysis of complex formation in heat-inactivated extracts from prp8 mutant strains may provide clues as to the domains of PRP8 involved in certain interactions. To date, six different temperature sensitive alleles of PRP8 have been isolated and for only one of these, prp8-1, has the mutation been approximately mapped within the gene (S. Jackson, Ph.D. thesis).

With respect to the genetic approach, the isolation of cold-sensitive mutations which suppress the heat-sensitive defect of prp8 mutations constitutes the most direct route to analysis of the interactions of PRP8. Suppression of the defect caused by mutation in one gene by mutation in another strongly implies a direct interaction between the products of the respective genes during the execution of their function. By this means, the protein-encoding SPR81 gene, certain cold-sensitive alleles of which will suppress the defect of the prp8-1 mutation, has been cloned and work is under way to raise antisera to fusion proteins expressed from it (D.Jamieson, personal communication). These antisera should allow analysis of the putative interaction between the proteins in the splicing process.

It should be possible to subsequently isolate heat-sensitive suppressors of the cold-sensitive mutations of SPR81 and then further cold-sensitive suppressors of those ad infinitum. This approach will combine with that of screening banks of temperature sensitive mutants for new prp mutations to identify, genetically, many of the components of the yeast splicing machinery. Therefore, the powerful combination of biochemical and genetic approaches which are available in Saccharomyces cerevisiae probably provides the most direct route to elucidation of the detailed pathway of nuclear pre-mRNA splicing.

7.2 METAZOAN HOMOLOGUES OF PRP8

Unequivocal proof of functional homology between the 220kD HeLa protein, whose identification is described in Chapter 6, and yeast PRP8 must, obviously, await further characterisation of the role of the yeast species in pre-mRNA splicing. However, evidence for such homology is provided by certain findings; (i) both are components of the U5 snRNP, (ii) the HeLa protein displays sequence-independent RNA-binding properties, as does the region of PRP8 present in TRP8.5, (iii) a large HeLa protein is cross-linked, like PRP8, to pre-mRNA in the spliceosome. The inability of anti-PRP8 antibodies to precipitate this protein from HeLa cell nuclear extracts makes an analysis of its role in splicing in these extracts very difficult.

The cDNA encoding this protein could be cloned using either of two approaches. Firstly, the affinity purified anti-PRP8 antibodies, which detect only a single species on Western blots of HeLa cell nuclear extract, could be used to screen a human

cDNA library in a λgt11 expression vector. Alternatively, the protein present in purified U5 snRNP fractions could be subjected to protein microsequencing and an oligonucleotide probe, synthesised on the basis of the data obtained, used to screen a cDNA library. The derived protein sequence of the human species may show strong similarity to PRP8, thus providing further evidence of homology. In addition, antibodies could be raised to the human protein which should allow a dissection of its role in the in vitro splicing reaction.

If the derived sequence of the human protein exhibited short regions of more pronounced similarity to the yeast species, then these conserved motifs would be strong candidates for functionally important elements in PRP8. These would then be likely targets for site-directed mutagenesis of PRP8. If an in vitro mutagenesis approach was be taken to analyse this huge protein, such indications of important regions would be essential.

Analysis of the protein identified in D.melanogaster cell extracts by immunoblotting is at a very preliminary stage and any suggestion of functional homology must await demonstration that it is a snRNP component. This system could prove invaluable if this protein is, indeed, a snRNP protein and is, unlike the HeLa species, precipitable by anti-PRP8 antibodies. Functional analysis of the protein could then be undertaken in a Drosophila in vitro splicing system, such as that recently described (Rio, 1988). Cloning and sequencing of the cDNA encoding the Drosophila protein would, as for the human case, provide insight into the functionally significant regions of PRP8.

In conclusion, the conservation of epitopes in three distinct regions of the molecule, and also conservation of its large size, suggest a central role for the PRP8 protein, and its homologues, in the pre-mRNA splicing process. To date, there have been no reports of the identification of metazoan homologues of any other yeast splicing proteins. The combination of approaches outlined in this Chapter should prove instrumental in the elucidation of the function of this species.

REFERENCES

Abelson J (1979) Ann Rev Biochem 48, 1035-1069.

Aebi M, Hornig H, Padgett RA, Reiser J, Weissmann C (1986) Cell 47, 555-565.

Aebi M, Hornig H, Weissmann C (1987) Cell 50, 237-246.

Amaldi F, Bozzoni I, Beccari E, Pierandrei-Amaldi P (1989) TIBS 14, 175-178.

Ares M, Jr (1986) Cell 47, 49-59.

Bach M, Winkelmann G, Luhrmann R (1989) Proc Natl Acad Sci USA (in press).

Banroques J, Abelson J (1989) Mol Cell Biol (in press).

Beggs JD, van den Berg J, van Ooyen A, Weissmann C (1980) Nature 283, 835-840.

Bektesh S, van Doren K, Hirsh D (1988) Genes Dev 2, 1277-1293.

Berget SM, Robberson BL (1986) Cell 46, 691-696.

Bindereif A, Green MR (1986) Mol Cell Biol 6, 2582-2592.

Bindereif A, Green MR (1987) EMBO J 6, 2415-2424.

Birnboim HC, Doly J (1979) Nucleic Acids Res 7, 1513-1523.

Black DL, Steitz JA (1986) Cell 46, 697-704.

Black DL, Chabot B, Steitz JA (1985) Cell 42, 737-750.

Blumenthal T, Thomas J (1988) TIG 4, 305-308.

Bornstein P, McKay J (1988) J Biol Chem 263, 1603-1606.

Botstein D, Falco SC, Stewart SE, Brennan M, Scherer S, Stinchcomb DT, Struhl K, Davis RW (1979) Gene 8, 17-24.

Boyer HW, Roulland-Dussoix D (1969) J Mol Biol 41, 459-472.

Bozzoni I, Annesi F, Beccari E, Fragapane P, Pierandrei-Amaldi P, Amaldi F (1984) J Mol Biol 180, 1173-1178.

Branlant C, Krol A, Ebel JP, Lazar E, Haendler B, Jacob M (1982) EMBO J 1, 1259-1265.

Breathnach R, Chambon P (1981) Ann Rev Biochem 50, 349-383.

Breitbart RE, Nadal-Ginard B (1987) Cell 49, 793-803.

Breitbart RE, Nguyen HT, Medford RM, Destree AT, Mahdavi V, Nadal-Ginard B (1985) Cell 41, 67-82.

Breitbart RE, Andreadis A, Nadal-Ginard B (1987) Ann Rev Biochem 56, 467-495.

Brennwald P, Porter G, Wise JA (1988) Mol Cell Biol 8, 5575-5580.

Bringmann P, Lührmann R (1986) EMBO J 5, 3509-3516.

Bringmann P, Rinke J, Appel B, Reuter R, Lührmann R (1983) J Biol Chem 258, 2745-2747.

Bringmann P, Appel B, Rinke J, Reuter R, Theissen H, Lührmann R (1984) EMBO J 1, 1259-1265.

Brody E, Abelson J (1985) Science 228, 963-967.

Brow DA, Guthrie C (1988) Nature 334, 213-218.

Bruzik JP, van Doren K, Hirsh D, Steitz JA (1988) Nature 335, 559-562.

Buchman AR, Berg P (1988) Mol Cell Biol 8, 4395-4405.

Bech TR (1986) Proc Natl Acad Sci USA 83, 4360-4363.

Cech TR (1987) Science 236, 1532-1539.

Cech TR, Bass BL (1986) Ann Rev Biochem 55, 599-629.

Cellini A, Parker R, McMahon J, Guthrie C, Rossi J (1986a) Mol Coll Biol 6, 1571-1578.

Cellini A, Felder E, Rossi JJ (1986b) EMBO J 5, 1023-1030.

Chabot B, Black DL, Lemaster DM, Steitz JA (1985) Science 230, 1344-1349.

Chabot B, Steitz JA (1987a) Mol Cell Biol 7, 698-707.

Chabot B, Steitz JA (1987b) Mol Cell Biol 7, 281-293.

Chang T-H, Clark MW, Lustig AJ, Cusick ME, Abelson J (1988) Mol Cell Biol 8, 2379-2393.

Cheng S-C, Abelson J (1986) Proc Natl Acad Sci USA 83, 2387-2391.

Cheng S-C, Abelson J (1987) Genes Dev 1, 1014-1027.

Choi YD, Grabowski PJ, Sharp PA, Dreyfuss G (1986) Science 231, 1534-1539.

Chou T-B, Zachar Z, Bingham PM (1987) EMBO J 6, 4095-4104.

Church GM, Gilbert W (1984) Proc Natl Acad Sci USA 81, 1991-1995.

Couto JR, Tamm J, Parker R, Guthrie C (1987) Genes Dev 1, 445-455.

Dabeva MD, Post-Beitenmiller MA, Warner JR (1986) Proc Natl Acad Sci USA 83, 5854-5857.

Dahlberg JE, Lund E (1988) in "Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles", Max L Birnsteil (ed), pp 38-70. Springer-Verlag, Berlin.

Davanloo P, Rosenberg AH, Dunn JJ, Studier FW (1984) Proc Natl Acad Sci USA 81, 2035.

Das G, Henning D, Wright D, Reddy R (1988) EMBO J 7, 503-512.

Dente L, Cesarini G, Cortese R (1983) Nucleic Acids Res 11, 1645-1655.

Dieckmann CL, Tzagoloff A (1985) J Biol Chem 260, 1513-1520.

Domdey H, Apostol B, Lin R-J, Newman A, Brody E, Abelson J (1984) Cell 39, 611-621.

Doolittle WF (1978) Nature 272, 581-582.

Dreyfuss G, Swanson MS, Piñol-Roma S (1988) TIBS 13, 86-91.

Duchêne M, Löw A, Schweizer A, Domdey H (1988) Nucleic Acids Res 16, 7233-7239.

England TE, Bruce AG, Uhlenbeck OC (1980) in "Methods in Enzymology", Grossman L, Moldave K (eds) pp 65-74. Academic Press, New York.

Eperon LP, Estibeiro JP, Eperon IC (1986) Nature 324, 280-282.

Eperon LP, Graham, IR, Griffiths AD, Eperon IC (1988) Cell 54, 393-401.

Feinberg AP, Vogelstein B (1984) Anal Biochem 137, 266-267.

Fink GR (1987) Cell 49, 5-6.

Fisher DE, Conner GE, Reeves NH, Wisniewolski R, Blobel G (1985) Cell 42, 751-758.

Folk WR (1988) Genes Dev 2, 373-375.

Forbes DJ, Kirschner MW, Caput D, Dahlberg JE, Lund E (1984) Cell 38, 681-689.

Fouser LA, Friesen JD (1986) Cell 45, 81-93.

Fouser LA, Friesen JD (1987) Mol Cell Biol 7, 225-230.

Fradin A, Jove R, Hemenway C, Keiser HD, Manley JL, Prives C (1984) Cell 37, 927-936.

Frendeway D, Keller W (1985) Cell 42, 355-367.

Furneaux HM, Perkins KK, Freyer GA, Arenas J, Hurwitz J (1985) Proc Natl Acad Sci USA 82, 4351-4355.

Gallwitz D (1982) Proc Natl Acad Sci USA 79, 3493-3497.

Garber RL, Kuroiwa A, Gehring WJ (1983) EMBO J 2, 2027-2036.

Geiduschek EP, Tocchini-Valentini GP (1988) Ann Rev Biochem 57, 873-914.

Gerke V, Steitz JA (1986) Cell 47, 973-984.

Ghosh PK, Reddy VB, Swinscoe J, Lebovitz P, Weissmann SM (1978) J Mol Biol 126, 813-846.

Gilbert W (1978) Nature 271, 501.

Gilbert W (1985) Science 228, 823-824.

Gilbert W (1986) Nature 319, 618.

Gilbert W, Marchionni M, McKnight G (1986) Cell 46, 151-154.

Goldenberg CJ, Hauser SD (1983) Nucleic Acids Res 11, 1337-1348.

Goldenberg CJ, Raskas HJ (1981) Proc Natl Acad Sci USA 78, 5430-5434.

Grabowski PJ, Sharp PA (1986) Science 233, 1294-1299.

Grabowski PJ, Padgett RA, Sharp PA (1984) Cell 37, 415-427.

Grabowski PJ, Seilor SR, Sharp PA (1985) Cell 42, 345-353.

Green MR (1986) Ann Rev Genet 20, 671-708.

Grunstein M, Hogness DS (1975) Proc Natl Acad Sci USA 72, 3961-3965.

Gudenus R, Mariotte S, Moenne A, Ruet A, Memet S, Buhler J-M, Sentenac A, Thuriaux P (1988) Genetics 119, 517-526.

Guerry P, Leblanc DJ, Falkow S (1973) J Bacteriol 116, 1064-1066.

Guthrie C, Riedel N, Parker R, Swerdlow H, Patterson B (1986) In "Yeast Cell Biology", UCLA Symposia on Molecular and Cellular Biology, New Series, Vol 33, Hicks J (ed) pp 301-321. Alan R Liss, New York.

Habets WJ, Sillekens PTG, Hoet MH, Schalken JA, Roebroek AJM, Leunissen JAM, van den Ven WJM, van Venrooij WJ (1987) Proc Natl Acad Sci USA 84, 2421-2425.

Hall R, Hyde JE, Goman M, Simmons DL, Hope IA, Mackay M, Scaife J (1984) Nature 311, 379-382.

Hamm J, Kazmaier M, Mattaj IW (1987) EMBO J 6, 3479-3485.

Hamm J, van Santen V, Spritz R, Mattaj I (1988) Proc Natl Acad Sci USA 8, 4787-4791.

Hanahan D (1985) in "DNA Cloning Volume I", Glover DM (ed) p 109-136. IRL Press, Oxford.

Hardy SF, Grabowski PJ, Padgett RA, Sharp PA (1984) Nature 308, 375-377.

Hartwell LH (1967) J Bacteriol 93, 1662-1670.

Hashimoto C, Steitz JA (1984) Nucleic Acids Res 12, 3283-3293.

Hernandez N, Keller W (1983) Cell 35, 89-99.

Hindley J, Phear GA (1984) Gene 31, 129-134.

Hinterberger M, Pettersson I, Steitz JA (1983) J Biol Chem 258, 2604-2613.

Hodgkin J (1989) Cell 56, 905-906.

Hornig H, Aebi M, Weissmann C (1986) Nature 324, 589-591.

Igel AH, Ares M, Jr (1988) Nature 334, 450-453.

Ito H, Fukada Y, Murada K, Kimura A (1983) J Bacteriol 153, 163-168.

Jackson SP, Lossky M, Beggs JD (1988) Mol Cell Biol 8, 1067-1075.

Jacob M, Gallinaro H (1989) Nucleic Acids Res 17, 2159-

Jacquier A, Rosbash M (1986) Proc Natl Acad Sci USA 83, 5835-5839.

Jacquier A, Rodriguez JR, Rosbash M (1985) Cell 43, 423-430.

Kaufer NF, Simanis V, Nurse P (1985) Nature 318, 78-80.

Kedes DH, Steitz JA (1988) Genes Dev 2, 1448-1459.

Keller EB, Noon WA (1984) Proc Natl Acad Sci USA 81, 7417-7420.

Keller EB, Noon WA (1985) Nucleic Acids Res 13, 4971-4981.

Kinlaw CS, Robberson BL, Berget SM (1983) J Biol Chem 258, 7181-7189.

Klinz F-J, Gallwitz D (1985) Nucleic Acids Res 13, 3791-3804.

Köhrer K, Domdey H (1988) Nucleic Acids Res 16, 9457-9476.

Kole R, Weissman SM (1982) Nucleic Acids Res 10, 5429-5445.

Konarska MM, Sharp PA (1986) Cell 46, 845-855.

Konarska MM, Sharp PA (1987) Cell 49, 763-774.

Konarska MM, Sharp PA (1988) Proc Natl Acad Sci USA 85, 5459-5462.

Konarska MM, Grabowski PJ, Padgett RA, Sharp PA (1985a) Nature 313, 552-557.

Konarska MM, Padgett RA, Sharp PA (1985b) Cell 42, 165-171.

Korf GM, Botros IW, Stumph WE (1988) Mol Cell Biol 8, 5566-5569.

Krainer AR, Maniatis T (1985) Cell 42, 725-736.

Krainer AR, Maniatis T, Ruskin B, Green MR (1984) Cell 36, 993-1005.

Krämer A (1988) Genes Dev 2, 1155-1167.

Krämer A, Keller W (1985) EMBO J 4, 3571-3581.

Krämer A, Keller W, Appel B, Lührmann R (1984) Cell 38, 299-307.

Krämer A, Frick M, Keller W (1987) J Biol Chem 262, 17630-17640.

Kretzner L, Rymond BC, Rosbash M (1987) Cell 50, 593-602.

Kunkel GR, Maser RL, Calvet JP, Pederson T (1986) Proc Natl Acad Sci USA 83, 8575-8579.

Laemmli UK (1970) Nature 227, 680-685.

Lambowitz AM (1989) Cell 56, 323-326.

Lamond AI, Konarska MM, Sharp PA (1987) Genes Dev 1, 532-543.

Lamond A, Konarska M, Grabowski P, Sharp P (1988) Proc Natl Acad Sci USA 85, 411-415.

Langford CJ, Gallwitz D (1983) Cell 33, 519-527.

Langford CJ, Klinz F-J, Donath C, Gallwitz D (1984) Cell 36, 645-653.

Laskey RA (1980) in "Methods in Enzymology" Grossman L, Moldave K (eds) pp 363-370. Academic Press, New York.

Laski FA, Rio DC, Rubin GM (1986) Cell 44, 7-19.

Last RL, Woolford JL, Jr (1986) J Cell Biol 103, 2103-2112.

Last RL, Stavenhagen JB, Woolford JL, Jr (1984) Mol Cell Biol 4, 2396.

Lee MG, Young RA, Beggs JD (1984) EMBO J 3, 2825-2830.

Lee MG, Lane DP, Beggs JD (1986) Yeast 2, 59-67.

Leer RJ, van Ramsdonk-Duin MM, Hagendoorn MJ, Mager WH, Planta R (1984) Nucleic Acids Res 12, 6685-6700.

Legrain P, Seraphin B, Rosbash M (1988) Mol Cell Biol 8, 3755-3760.

Lerner MR, Boyle JA, Mount SM, Wolin SL, Steitz JA (1980) Nature 283, 220-224.

Liautard JP, Sri-Widada J, Brunel C, Jeanteur P (1982) J Mol Biol 162, 623-643.

Lin R-J, Newman AJ, Cheng S-C, Abelson J (1985) J Biol Chem 260, 14780-14792.

Lin R-J, Lustig AJ, Abelson J (1987) Genes Dev 1, 7-18.

Lossky M, Anderson GJ, Jackson SP, Beggs JD (1987) Cell 51, 1019-1026.

Lowery DE, van Ness BG (1988) Mol Cell Biol 8, 2610-2619.

Lührmann R (1988) in "Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles", Birnstiel ML (ed) pp 71-99. Springer-Verlag, Berlin.

Lund E, Dahlberg JE (1987) Genes Dev 1, 39-46.

Lund E, Kahan B, Dahlberg JE (1985) Science 229, 1271-1274.

Lund E, Bostock CJ, Dahlberg JE (1987) Genes Dev 1, 47-56.

Lustig AJ, Lin R-J, Abelson J (1986) Cell 47 953-963.

Maniatis T, Fritsch TF, Sambrook J (1982) in "Molecular Cloning". Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mattaj I (1986) Cell 46, 905-911.

Mattaj I (1989) Cell 57, 1-3.

Mattaj IW, Dathan NA, Parry HD, Carbon C, Krol A (1988) Cell 55, 435-442.

Maxam A, Gilbert W (1980) in "Methods in Enzymology", Vol 65, Grossman L, Moldave K (eds) p 499. Academic Press, New York.

Mayeda A, Tatei K, Kitayama H, Takemura K, Ohshima Y (1986) Nucleic Acids Res 14, 3045-3057.

McAllister G, Amara SG, Lerner MR (1988) Proc Natl Acad Sci USA 85, 5296-5300.

McAllister G, Roby-Shemkovitz A, Amara SG, Lerner MR (1989) EMBO J 8, 1177-1181.

Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR (1984) Nucleic Acids Res 12, 7035-7056.

Messing J, Gronenborn B, Muller-Hill B, Hofschneider PH (1977) Proc Natl Acad Sci USA 74, 3642-3646.

Miller AM (1984) EMBO J 3, 1061-1065.

Morrissey JE (1981) Anal Biochem 117, 307-310.

Mottram J, Perry KL, Lizardi PM, Lührmann R, Agabian N, Nelson RG (1989) Mol Cell Biol 9, 1212-1223.

Mount SM (1982) Nucleic Acids Res 10, 459-472.

Mount SM, Pettersson I, Hinterberger M, Karmas A, Steitz JA (1983) Cell 33, 509-518.

Murphy WJ, Watkins KP, Agabian N (1986) Cell 47, 517-525.

Nelson KK, Green MR (1988) Genes Dev 2, 319-329.

Newman A (1987) EMBO J 6, 3833-3839.

Newman AJ, Lin R-J, Cheng S-C, Abelson J (1985) Cell 42, 335-344.

Ng R, Domdey H, Larson G, Rossi JJ, Abelson J (1985) Nature 314, 183-184.

Noble JCS, Prives C, Manley JL (1986) Nucleic Acids Res 14, 1219-1235.

Noble JCS, Prives C, Manley JL (1988) Genes Dev 2, 1460-1475.

Orgel LE, Crick FHC (1980) Nature 284, 604-607.

Padgett RA, Hardy SF, Sharp PA (1983) Proc Natl Acad Sci USA 80, 5230-5234.

Padgett RA, Mount SM, Steitz JA, Sharp PA (1983) Cell 35, 101-107.

Padgett RA, Konarska MM, Grabowski PJ, Hardy SF, Sharp PA (1984) Science 225, 898-903.

Padgett RA, Grabowski PJ, Konarska MM, Seilor S, Sharp PA (1986) Ann Rev Biochem 55, 1119-1150.

Parent A, Zeitlin S, Efstradiatis A (1987) J Biol Chem 262, 11284-11291.

Parker R, Guthrie C (1985) Cell 41, 107-118.

Parker R, Siliciano PG, Guthrie C (1987) Cell 49, 229-239.

Parker R, Simmons T, Shuster EO, Siliciano PG, Guthrie C (1988) Mol Cell Biol 8, 3150-3159.

Parry HD, Scherly D, Mattaj IW (1989) TIBS 14, 15-19.

Patterson B, Guthrie C (1987) Cell 49, 613-624.

Patton JR, Pederson T (1988) Proc Natl Acad Sci USA 85, 747-751.

Patton JR, Patterson RJ, Pederson T (1987) Mol Cell Biol 7, 4030-4037.

Perkins KK, Furneaux HM, Hurwitz J (1986) Proc Natl Acad Sci USA 83, 887-891.

Perler F, Efstradiatis A, Lomedico P, Gilbert W, Kolodner R, Dodgson J (1980) Cell 20, 555-566.

Peterson-Bjorn S, Soltyk A, Beggs JD, Friesen JD (1989) Mol Cell Biol (in press).

Pheiffer BH, Zimmermann SB (1983) Nucleic Acids Res 11, 7853-7871.

Pikielny CW, Rosbash M (1985) Cell 41, 119-126.

Pikielny CW, Rosbash M (1986) Cell 45, 869-877.

Pikielny CW, Teem JL, Rosbash M (1983) Cell 34, 395-403.

Pikielny CW, Rymond BC, Rosbash M (1986) Nature 324, 341-345.

Potashkin J, Li R, Frendeway D (1989) EMBO J 8, 551-559.

Query CC, Bentley RC, Keene JD (1989) Cell 57, 89-101.

Rautmann G, Matthes HWD, Gait MJ, Breathnach R (1984) EMBO J 3, 2021-2028.

Reddy R, Henning D, Das G, Harless M, Reddy R (1987) J Biol Chem 262, 75-81.

Reed R, Maniatis T (1985) Cell 41, 95-105.

Reed R, Maniatis T (1986) Cell 46, 681-690.

Reed R, Maniatis T (1988) Genes Dev 2, 1268-1276.

Reed R, Griffith J, Maniatis T (1988) Cell 53, 949-961.

Reuter R, Rothe S, Lührmann R (1987) Nucleic Acids Res 15, 4021-4034.

Riedel N, Wise JA, Swerdlow H, Mak A, Guthrie C (1986) Proc Natl Acad Sci USA 83, 8097-8101.

Riedel N, Wolin S, Guthrie C (1987) Science 235, 328-331.

Rinke J, Appel B, Digweed M, Lührmann R (1985) J Mol Biol 185, 721-731.

Rio DC (1988) Proc Natl Acad Sci USA 85, 2904-2908.

Robinson PA, Anderton BH, Loviny TLF (1988) J Immunol Meth 108, 115-122.

Rodriguez JR, Pikielny CW, Rosbash M (1984) Cell 39, 603-610.

Rogers J, Wall R (1980) Proc Natl Acad Sci USA 77, 1877-1879.

Rokeach LA, Haselby JA, Hoch SO (1988) Proc Natl Acad Sci USA 85, 4832-4836.

Rosbash M, Harris PKW, Woolford JL, Jr, Teem JL (1981) Cell 24, 679-686.

Ruby SW, Abelson J (1988) Science 242, 1028-1035.

Ruskin B, Green MR (1985a) Nature 317, 732-734.

Ruskin B, Green MR (1985b) Cell 43, 131-142.

Ruskin B, Krainer AR, Maniatis T, Green MR (1984) Cell 38, 317-331.

Ruskin B, Greene JM, Green MR (1985) Cell 41, 833-844.

Ruskin B, Pikielny CW, Rosbash M, Green MR (1986) Proc Natl Acad Sci USA 83, 2022-2026.

Ruskin B, Zamore PD, Green MR (1988) Cell 72, 207-219.

Ruther U, Muller-Hill B (1983) EMBO J 2, 1791-1794.

Rymond BC, Rosbash M (1985) Nature 317, 735-737.

Rymond BC, Rosbash M (1986) EMBO J 5, 3517-3524.

Rymond BC, Rosbash M (1988) Genes Dev 2, 428-439.

Rymond BC, Torrey DD, Rosbash M (1987) Genes Dev 1, 238-246.

Sanger F, Coulson AR (1978) FEBS Lett 7, 107-110.

Sanger F, Nicklen S, Coulson AR (1977) Proc Natl Acad Sci USA 74, 5463-5476.

Schmidt RJ (1985) Cell 41, 339-340.

Scott MP, Weiner AJ, Hazelrigg TI, Polisky BA, Pirrotta V, Scalenghe F, Kaufmann TC (1983) Cell 35, 763-776.

Séraphin B, Kretzner L, Rosbash M (1988) EMBO J 7, 2533-2538.

Sharp PA (1987a) Science 235, 766-771.

Sharp PA (1987b) Cell 50, 147-148.

Sherman F, Fink GR, Hicks JB (1983) in "Methods in Yeast Genetics". Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Shih M-C, Heinrich P, Goodman HM (1988) Science 242, 1164-1166.

Shuster EO, Guthrie C (1988) Cell 55, 41-48.

Siliciano PG, Guthrie C (1988) Genes Dev 2, 1258-1267.

Siliciano PG, Brow DA, Roiha H, Guthrie C (1987a) Cell 50, 585-592.

Siliciano PG, Jones MH, Guthrie C (1987b) Science 237, 1484-1487.

Sillekens PTG, Habets WJ, Beijer RP, van Venrooij WJ (1987) EMBO J 6, 3841-3848.

Sillekens PTG, Beijer RP, Habets WJ, van Venrooij WJ (1988) Nucleic Acids Res 15, 10373-10391.

Sillekens PTG, Beijer RP, Habets WJ, van Venrooij WJ (1989) Nucleic Acids Res 17, 1893-1906.

Smith JH, Eliceiri GL (1983) J Biol Chem 258, 4636-4640.

Sollner-Webb B (1988) Cell 52, 153-154.

Solnick D (1985) Cell 42, 157-164.

Soltyk A, Tropak M, Friesen JD (1984) J Bacteriol 160, 1093-1100.

Southern EM (1975) J Mol Biol 98, 503-517.

Spindler KR, Rosser DSE, Berk AJ (1984) J Virology 49, 132-141.

Stanley KK, Luzio JP (1984) EMBO J 3, 1429-1434.

Steitz JA (1988) Sci Amer 258, 36-41.

Steitz JA, Black DL, Gerke V, Parker KA, Krämer A, Frendeway D, Keller W (1988) in "Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles", Birnstiel ML (ed) pp 115-154. Springer-Verlag, Berlin.

Sudhof TC, Russel DW, Goldstein JL, Brown MS, Sanchez-Pescador R, Bell GI (1985) Science 228, 893-895.

Sutton RE, Boothroyd JC (1986) Cell 47, 527-535.

Swanson MS, Dreyfuss G (1988a) Mol Cell Biol 8, 2237-2241.

Swanson MS, Dreyfuss G (1988b) EMBO J 7, 3519-3529.

Tazi J, Alibert C, Temsamani J, Reveillaud I, Cathala G, Brunel C, Jeanteur P (1986) Cell 47, 755-766.

Temsamani J, Alibert C, Tazi J, Rucheton M, Capony J-P, Jeanteur P, Cathala G, Brunel C (1989) J Mol Biol 206, 439-449.

Theissen H, Etzerodt M, Reuter R, Schneider C, Lottspeich F, Argos P, Lührmann R, Philipson L (1986) EMBO J 5, 3209-3217.

Thomas JD, Conrad RC, Blumenthal T (1988) Cell 54, 533-539.

Thompson-Jäger S, Domdey H (1987) Mol Cell Biol 7, 4010-4016.

Tollervey D (1987) J Mol Biol 196, 355-361.

Tollervey D, Mattaj IW (1987) EMBO J 6, 469-476.

Towbin H, Staehelin T, Gordon J (1979) Proc Natl Acad Sci USA 76, 4350-4354.

Treisman R, Novak J, Favaloro J, Kamen R (1981) Nature 292, 595-600.

Treisman R, Orkin SH, Maniatis T (1983) Nature 302, 591-596.

Turnbull-Ross AD, Else AJ, Eperon IC (1988) Nucleic Acid Res 16, 395-411.

van der Ploeg (1986) Cell 47, 479-480.

van Doren K, Hirsh D (1988) Nature 335, 556-559.

van Santen VL, Spritz RA (1985) Proc Natl Acad Sci USA 82, 2885-2889.

van Venrooij WJ (1987) J Rheumatol 14, 78-82.

Vijayraghavan U, Parker R, Tamm J, Iimura Y, Rossi J, Abelson J, Guthrie C (1986) EMBO J 5, 1683-1695.

Wallace JC, Edmonds M (1983) Proc Natl Acad Sci USA 80, 950-954.

Weber S, Aebi M (1988) Nucleic Acids Res 16, 471-486.

Weingärtner B, Keller W (1981) Proc Natl Acad Sci USA 78, 4092-4096.

Weiringa B, Meyer F, Reiser J, Weissmann C (1983) Nature 301, 38-43.

Weiringa B, Hofer E, Weissmann C (1984) Cell 37, 915-925.

Wieczorek DF, Smith CWJ, Nadal-Ginard B (1988) Mol Cell Biol 8, 679-694.

Wieben ED, Rohleder AM, Nenninger JM, Pederson T (1985) Proc Natl Acad Sci USA 82, 7914-7918.

Wise JA, Tollervey D, Maloney D, Swerdlow H, Dunn EJ, Guthrie C (1983) Cell 35, 743-757.

Witkowski JA (1988) TIBS 13, 110-113.

Woppmann A, Rinke J, Lührmann R (1988) Nucleic Acids Res 16, 10985-11004.

Wozney J, Hanahan D, Tate V, Boedtker H, Doty P (1981) Nature 294, 129-135.

Yang VW, Lerner MR, Steitz JA, Flint SJ (1981) Proc Natl Acad Sci USA 78, 1371-1375.

Zachar Z, Chou T-B, Bingham PM (1987) EMBO J 6, 4105-4111.

Zapp ML, Berget SM (1989) Nucleic Acids Res 17, 2655-

Zaug AJ, Cech TR (1986) Science 231, 470-475.

Zeitlin S, Efstradiatis A (1984) Cell 39, 589-602.

Zhuang Y, Weiner AM (1986) Cell 46, 826-835.

Zillmann M, Rose SD, Berget SM (1987) Mol Cell Biol 7, 2877-2883.

Zillmann M, Zapp ML, Berget SM (1988) Mol Cell Biol 8, 814-821.

APPENDIX

EVIDENCE THAT THE SNR6 GENE IS TRANSCRIBED BY RNA POLYMERASE C

A.1 INTRODUCTION

The majority of snRNAs possess a characteristic trimethyl guanosine cap structure and are transcribed by RNA polymerase B (RNA polymerase II). Transcription of the U6 gene, however, can be achieved in cell-free extracts lacking polymerase B activity, is relatively insensitive to α-amanitin and can be competed by excess copies of the class C 5S rRNA gene (Kunkel et al., 1986; Reddy et al., 1987). This evidence suggests that the U6 gene is transcribed by RNA polymerase C (III).

In contrast to the use of internal promoters found in other class C genes (Geiduschek and Tocchini-Valentini, 1988), upstream regions alone prove sufficient for efficient transcription of a U6 gene in vitro (Das et al., 1988). The upstream promoter region shares sequence elements with pol B promoters (Parry et al., 1989). It has two conserved regions, the distal and proximal sequence elements, in common with, and functionally interchangeable with, the promoters of pol B-transcribed U snRNA genes. It also possesses a "TATA" box, a feature of many mRNA pol B promoters, but which in this case has been shown to be the element necessary to confer putative pol C transcription upon the U6 gene (Mattaj et al., 1988). Taken together these data suggest that there is not such a clear cut distinction between class B and class C genes as was once thought and raise the possibility that there exists a hybrid pol B/C which acts to transcribe the U6 gene.

This Appendix describes the direct genetic approach taken to analyse the possible involvement of pol C in the transcription of the yeast SNR6 gene (as a collaboration with the laboratory of A.Sentenac, C.E.N. de Saclay). For this, use was made of mutant strains of yeast that carry temperature sensitive defects in the largest subunit (RPC160) of RNA polymerase C, resulting in a dramatic reduction in tRNA synthesis at the non-permissive temperature (Gudenus et al., 1988).

A.2 U6 snRNA IS DEPLETED IN A rpc160 MUTANT

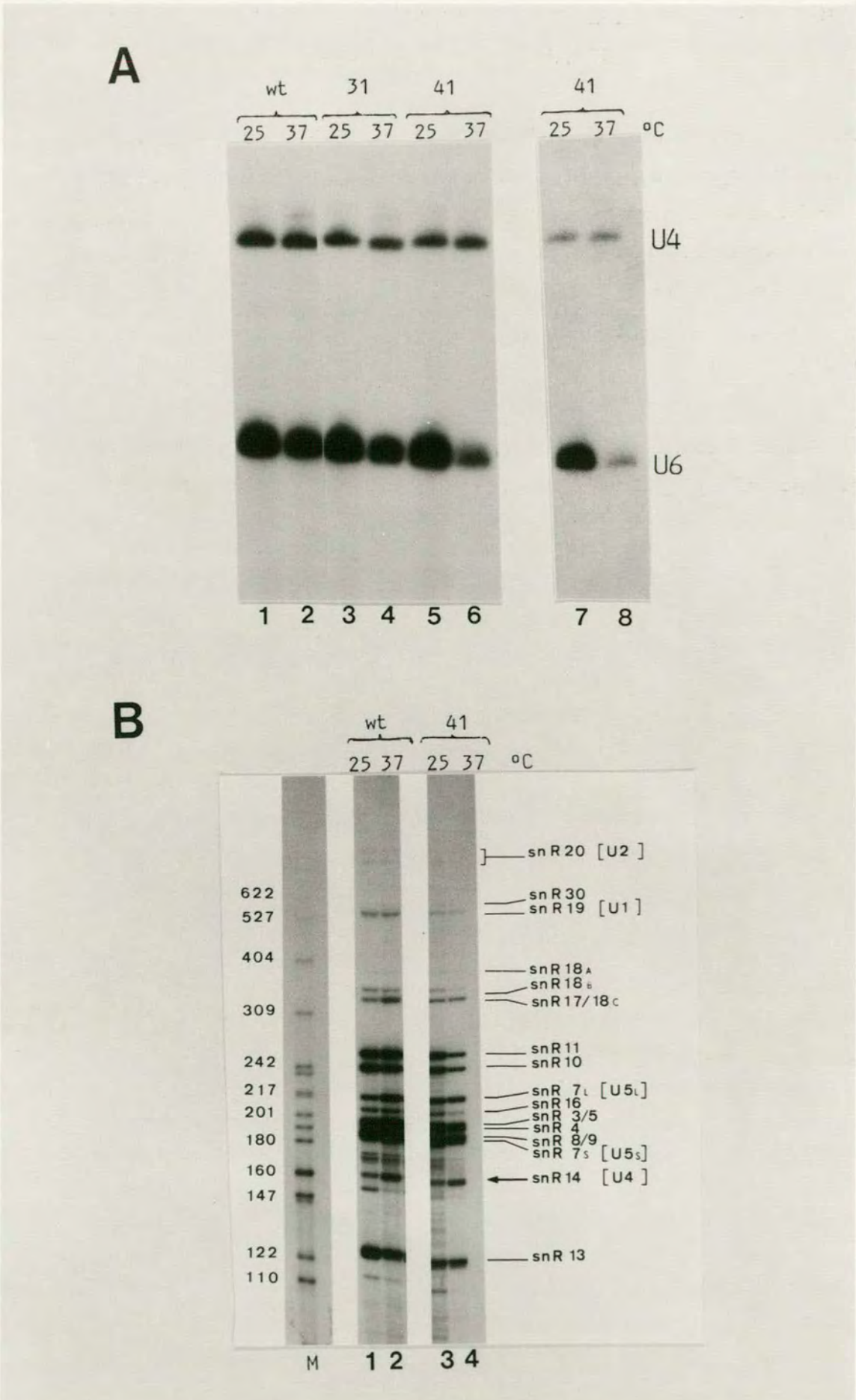
Cells of wild-type strain YNN281 and of mutant strains YNN281-31 (rpc160-31 temperature sensitive mutation) and YNN281- 41 (rpc160-41) were grown at 25°C and the cultures split, half being incubated at 25°C for a further 20 hours and the other half at 37°C for the same period, and total RNA was prepared (this procedure was performed by A.Moenne, C.E.N. de Saclay). The levels of U4 and U6 snRNAs were analysed on a Northern blot using oligonucleotide probes (Figure A.1A). Approximately equal loading of RNA from the different RNA preparations was achieved by comparison of the 28S and 18S rRNA bands on an ethidium bromide-stained agarose gel (data not shown) and the U4- and U6-specific signals were compared by densitometric analysis of the autoradiograph. The U4 snRNA levels were similar at the two growth temperatures in the wild-type and mutant strains. When the densitometric readings were corrected for the slight variation in the levels of U4 snRNA, it was seen that the U6 snRNA level was depleted during growth at the higher temperature by 21% in wild-type cells (lanes 1 and 2), by 28% in rpc160-31 cells (lanes 3 and 4) and by 80% in rpc160-41 cells (lanes 5 and 6 and more clearly seen with a shorter exposure, lanes 7 and 8). A blot probed with a U2 snRNA-specific oligonucleotide probe showed no significant effect of growth temperature on this species in any of the strains (data not shown). Analysis of RNA from rpc160-41 cells shifted to 37°C for 5 hours revealed a much less severe depletion of U6 snRNA (data not shown).

In order to compare the levels of a wider range of snRNAs in rpc160-41 cells, immunoprecipitations were performed using anti-m^3G antibodies and the precipitated RNA species were 3'-end labelled and analysed by denaturing polyacrylamide gel electrophoresis (Figure A.1B). There is little quantitative difference in any of the snRNAs, for the wild-type or rpc160-41 mutant strains, between cells grown at 25°C or shifted to 37°C (compare lanes 1 and 2 with lanes 3 and 4, respectively). This is consistent with the major m^3G-capped snRNA species being transcribed by RNA polymerase B (Dahlberg and Lund, 1988) and demonstrates that the rpc160-41 mutation has no general effect on snRNA levels at the non-permissive temperature.

FIGURE A.1: U6 snRNA IS SPECIFICALLY DEPLETED IN A POLYMERASE C MUTANT STRAIN

(A) Total RNA from wild-type cells (WT), grown at 25°C (lane 1) or 37°C (lane 2), from cells carrying the rpc160-31 mutation (31), grown at 25°C (lane 3) or 37°C (lane 4), and from cells carrying the rpc160-41 mutation (41), grown at 25°C (lane 5) or 37°C (lane 6), was separated on a 6% polyacrylamide/7M urea gel, electroblotted to Hybond N membrane and probed with 5'-end labelled oligonucleotides specific for U4 and U6 snRNAs. A shorter time exposure autoradiograph of lanes 5 and 6 is displayed in lanes 7 and 8.

(B) Anti-m^3G immunoprecipitations were performed on total RNA samples from wild-type and rpc160-41 cells, prepared as in (A). RNA recovered from immune complexes was 3'-end labelled and analysed by electrophoresis through a 6% polyacrylamide/7M urea gel. Markers were an end labelled MspI digest of pBR322 plasmid DNA.


A
wt
31
41
41
25 37 25 37 25 37 25 37 °C
U4
U6
1 2 3 4 5 6 7 8
B
wt
41
25 37 25 37 °C
622
527
404
309
242
217
201
180
160
147
122
110
snR 20 [U2]
snR 30
snR 19 [U1]
snR 18A
snR 18B
snR 17/18C
snR 11
snR 10
snR 7L [U5L]
snR 16
snR 3/5
snR 4
snR 8/9
snR 7S [U5S]
snR 14 [U4]
snR 13
M 1 2 3 4

A.3 DISCUSSION

The results described in Section A.2 indicate that, in cells carrying the rpc160-41 mutation, the steady state level of U6 snRNA is specifically reduced, providing direct genetic evidence to support previous proposals that RNA polymerase C is involved in synthesis of this species. The steady state level of U6 snRNA in cells carrying the rpc160-31 mutation is not significantly different from that in wild-type cells after 20 hours heat-shock. This suggests that the defect caused by this particular mutation is in the interaction of RNA polymerase C with components necessary for the transcription of tRNA genes but not SNR6.

There remain two other possible explanations for these results. Firstly, and less likely, the U6 snRNA may be selectively degraded in the rpc160-41 mutant strain. Alternatively, the SNR6 gene may be transcribed by another, as yet undefined, class of RNA polymerase which shares the RPC160 subunit with RNA polymerase C, e.g. a hybrid pol B/C as postulated in Section A.1. However, the SNR6 gene has now been transcribed in vitro by purified RNA polymerase C in the presence of partially purified TFIIIB (Camier and Sentenac, personal communication), providing the first direct biochemical evidence for the involvement in U6 snRNA synthesis of the same RNA polymerase which acts to transcribe the class C genes with internal promoters. This argues against the existence of a hybrid RNA polymerase, but suggests that transcription factors are not specific to one class of genes, i.e class B and class C factors can interact in the formation of transcription initiation complexes.